DECLARATION

I, the below-named translator, hereby declare:

(1) That my name, mailing address and citizenship are as stated below;

(2) That I am knowledgeable in the English language and in the Korean language in which Korean Patent Application No. 10-2003-0038012 was filed on June 12, 2003; and

(3) That I have translated said Korean Patent Application No. 10-2003-0038012 into English, which English text is attached hereto, and believe that said translation is a true and complete translation of the aforementioned Korean patent application.

June 1, 2007

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KOREAN INDUSTRIAL PROPERTY OFFICE

This is to certify that the following application annexed hereto is a true copy from the records of the Korean Industrial Property Office.

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Date of Application: June 12, 2003

Applicant(s): PanGenomics Co., Ltd.

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FILING DETAILS OF THE PATENT APPLICATION

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[TITLE OF THE INVENTION]
Her-2/neu DNA VACCINE HAVING ANTI-CANCER ACTIVITY
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[NUCLEIC ACID SEQUENCE LISTINGS OR AMINO ACID SEQUENCE LISTINGS]
[NUMBER OF SEQUENCE] 24
[SEQUENCE LISTINGS ELECTRONIC FILE] ATTACHED

The above application is filed in accordance with Article 42 of Korean Patent Law, and the request for the examination of the above application is filed in accordance with Article 60 of Korean Patent Law.

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ABSTRACT

The present invention relates to a plasmid having anti-cancer effect, which comprises human Her-2/neu gene lacking the intracellular domain, and a composition and DNA vaccine comprising same for preventing or treating cancer. The Her-2/neu DNA vaccines of the present invention having excellent anti-cancer effect can be effectively used as a therapeutic vaccine in reducing metastasis after tumor surgery or as a prophylactic vaccine for people with genetic high risk.

Representative Figure

Fig. 1a

SPECIFICATION

Title of the Invention

Her-2/neu DNA VACCINE HAVING ANTI-CANCER ACTIVITY

Brief Description of the Drawings

Figs. 1a and 1b: schematic procedure for preparing pNeu plasmid constructs (1a) and a preset immunization schedule (1b);

ECD: extracellular domain,

TM: transmembrane domain,

ICD: intracellular domain

Fig. 2: Her-2/neu specific antibody response induced by pTV2 (A), $pNeu_{TM}$ (B), $pNeu_{ECD}$ (C), $pNeu_{TM}$-gDs (D) and $pNeu_{ECD}$-gDs (E) plasmids, respectively;

white histogram: control antibody,

black histogram: test antibody

Fig. 3: Her-2/neu specific antibody response induced by PBS (A), $pNeu_{ECD}$ (B), $pNeu_{TM}$ (C), pCK_{ECD} (D) and pCK_{TM} (E) plasmids, respectively;

white histogram: control antibody,

black histogram: test antibody

Fig. 4: confocal microscopic analysis of mouse sera immunized with pTV2 (A), $pNeu_{TM}$ (B) and $pNeu_{ECD}$-gDs (C) plasmids, respectively;

Fig. 5: cytotoxic T lymphocytes (CTL) responses induced by pTV2 (A), $pNeu_{TM}$ (B), $pNeu_{ECD}$ (C), $pNeu_{TM}$-gDs (D) and $pNeu_{ECD}$-gDs (E) plasmids, respectively;

Fig. 6: CTL responses induced by PBS (A), $pNeu_{ECD}$ (B), $pNeu_{TM}$ (C), pCK_{ECD} (D) and pCK_{TM} (E) plasmids, respectively;

Figs. 7a and 7b: preventive antitumor immunity induced by vaccination with pNeu plasmids with lapse of time; tumor size in mouse subcutaneously injected with Her2-CT26 cells after plasmid injection (7a), and survival rate in mouse intravenously injected with Her2-CT26 cells after plasmid injection (7b);

Figs. 8a and 8b: preventive antitumor immunity induced by vaccination with pCK_{ECD} and pCK_{TM} plasmids with lapse of time; tumor size in mouse subcutaneously injected with Her2-CT26 cells after plasmid injection (8a), and survival rate in mouse intravenously injected with Her2-CT26 cells after plasmid injection (8b);

Figs. 9a and 9b: preventive antitumor immunity induced by vaccination with $pNeu_{ECD}$ and $pNeu_{ECD}$-gDs with lapse of time; tumor size in mouse subcutaneously injected with Her2-CT26 cells after plasmid injection (9a), and survival rate in mouse intravenously injected with Her2-CT26 cells after plasmid injection (9b);

Fig. 10: therapeutic efficacy induced by vaccination with $pNeu_{ECD}$ and $pNeu_{ECD}$-gDs with lapse of time; survival rate in mouse intravenously injected with 1×10^5 Her2-CT26 cells (10a) and 5×10^5 Her2-CT26 cells (10b) followed by plasmid injection;

Fig. 11: therapeutic efficacies induced by vaccination with pCK_{ECD} and pCK_{TM};

Figs. 12a and 12b: vaccination schedule of co-injection with pCK_{TM} and cytokine plasmids (12a) and CTL responses induced thereby (12b), respectively;

Figs. 13a to 13d: preventive antitumor effect induced by co-injection with pCK_{TM} and cytokine plasmids; subcutaneous and intravenous injection schedule with Her2-CT26 cells after plasmid injection in mouse (13a), tumor size in subcutaneous injection model (13b, a parenthesis means the percentage of mouse having no tumor growth per treatment group), and survival rate in intravenous injection model (13c and 13d, a parenthesis means the percentage of live mouse per treatment group) with lapse of time;

Figs. 14a to 14c: therapeutic efficacies induced by co-injection with pCK_{TM} and pCK-cytokine plasmids; intravenous injection schedule with Her2-CT26 cells followed by plasmid injection in mouse (14a), and survival rate in intravenous injection model (14b and 14c, a parenthesis means the percentage of live mouse per treatment group) with lapse of time;

Figs. 15a to 15d: schematic procedure of constructing bicistronic plasmids co-expressing both Her-2/neu gene and cytokine gene (15a), and preventive antitumor effect induced by pCK_{TM}-cytokine plasmids; tumor size in

subcutaneous injection model of Her2-CT26 cells after plasmid injection (15b, a parenthesis means the percentage of mouse having no tumor growth per treatment group), and survival rate in intravenous injection model of Her2-CT26 cells (15c and 15d, a parenthesis means the percentage of live mouse per treatment group) with lapse of time;

Figs. 16a and 16b: therapeutic efficacies induced by plasmid co-expressing both Her-2/neu gene and cytokine gene (a parenthesis means the percentage of live mouse per treatment group).

Detailed Description of the Invention

Purpose of the Invention

Field of the Invention and Prior Arts

The present invention relates to a plasmid having anti-cancer effect, which comprises human Her-2/neu DNA, and a vaccine comprising same as an active ingredient.

The Her-2/neu encodes a transmembrane protein (185 kDa) that is a member of the type I family of growth factor receptors (Akiyama, T. et al., *Science* 232:1644-1646, 1986).

The Her-2/neu protein has been found to be amplified and overexpressed in several types of human adenocarcinomas, especially in tumors of the breast and the ovary. The overexpression of Her-2/neu was correlated with short relapse time and poor survival rate of breast cancer patients suggesting that Her-2/neu overexpression itself likely plays a critical role in the development of human cancers, and a direct role of Her-2/neu in the pathogenesis and clinical aggressiveness of Her-2/neu-expressing tumors (Slamon, D. J. et al., *Science* 235:177-182, 1987). Further, Her-2/neu-specific antibodies and T cells are detected in breast and ovarian cancer patients (Kobayashi, H., et al., *Cancer Res.*, 60:5228-5236, 2000). Therefore, Her-2/neu oncogene is an excellent target for the development of therapeutic vaccines specific for Her-2/neu-overexpressing cancers.

It is well known that human Her-2/neu gene has tyrosine kinase activity

in the intracellular domain and its overexpression itself stimulates abnormal cell division so that the possible oncogenecity is boosted. Therefore, there are several attempts to inhibit possible oncogenecity caused by using entire molecule in a vaccine by introducing a mutation into the cytoplasmic kinase active domain to inhibit tyrosine kinase activity or by using truncated molecules lacking the intracellular or extracellular domain (Wei, W. I. et al., *Int. J. Cancer* 81: 748-754, 1999).

Plasmids are relatively simple to generate and safe so that they can be attractive vectors for the development of anti-cancer vaccines encoding tumor-associated antigens. Because they are not proteins nor associated with a viral coat, they do not produce neutralizing antibodies that can hamper the clinical efficacy of vaccines.

In preclinical tumor models, DNA vaccines encoding rat Her-2/neu (Chen, Y. et al., *Cancer Res.*, 58:1965-1971, 1998) or human Her-2/neu (Pilon, S. A. et al., *J. Immunol.*, 167:3201-3206, 2001) induced preventive effect against Her-2/neu over-expressing cancer cells.

Although successful anti-cancer effects against Her-2/neu expressing cancer cells by Her-2/neu DNA vaccine have been achieved by many earlier experiments, no successful therapeutic effect has been reported. The difficulty lies on the slow gain of antitumor immunity due to the lag time before antigenic expression of Her-2/neu expressing plasmids, while breast cancer cell grows relatively fast. Therefore, some of the Her-2/neu therapeutic vaccine experiments were conducted by using the combination of DNA and cytokine-secreting cancer cells (Chen, S. A. et al., *Clin. Cancer Res.*, 6:4381-4388, 2000), or dendritic cell (Chen, Y., *Gene Ther.*, 8:316-323, 2001).

Therefore, the present inventors have endeavored to develop Her-2/neu DNA vaccines having high anti-cancer activity which can be effectively used as a DNA vaccine for preventing and treating cancer.

Technical Object of the Invention

Accordingly, it is an object of the present invention to provide a human Her-2/neu expressing plasmid construct having high antitumor activity.

It is another object of the present invention to provide a composition comprising the plasmid construct as an active ingredient for preventing and/or treating cancers.

It is further object of the present invention to provide a vaccine comprising the plasmid construct as an active ingredient for preventing and/or treating cancers.

Constitution of the Invention

In accordance with one aspect of the present invention, there is provided a plasmid construct having anti-cancer activity, which is prepared by inserting a truncated human Her-2/neu gene lacking the intracellular domain into pTV2 or pCK vector.

The truncated human Her-2/neu gene lacking the intracellular domain has the nucleotide sequence of SEQ ID NO: 2, and is inserted into pTV2 vector (Lee, S. W. et al., *J. Virol.*, 72:8430-8436, 1998) or pCK vector (Accession No.: KCCM-10179), which has a high expression level *in vivo*, preferably, into *Kpn*I/*Xba*I site (when the insert containing signal peptide sequence of Her-2/neu gene itself) or *Asc*I/*Xba*I site (when the insert containing herpes simplex virus type I glycoprotein D signal (gDs) sequence instead of the signal peptide sequence of HER-2/neu gene) of each vector.

In the present invention, deleting the intracellular domain from human Her-2/neu gene of the inventive plasmid has the following advantages. Namely, possible oncogenecity of Her-2/neu can be eliminated by constructing truncated Her-2/neu plasmids lacking the Her-2/neu cytoplasmic kinase domain to block the abnormal growth signal transduction caused by the cytoplasmic kinase domain. Further, the risks of chance transforming of normal cells and transmission of abnormal growth signal toward tumor malignancy that may be caused by tyrosine kinase in the intracellular domain. In addition, the truncated Her-2/neu of the present invention enables to avoid the dangers of autoimmunity against the Her-2/neu intracellular domain that is highly conserved among the members of the EGFR (epidermal growth factor receptor) family. DNA vaccines using the truncated Her-2/neu lacking the intracellular

domain in order to exclude the risks of oncogenecity and autoimmunity have been reported (Chen, Y. et al., *Cancer Res.*, 58:1965-1971, 1998), but they did not have excellent anti-cancer effect. Whereas, the inventive plasmid has excellent anti-cancer effect by inducing both antibody response and CTL response, and particularly shows therapeutic effect against metastatic cancer.

The present invention also provides a plasmid constructs encoding the truncated human Her-2/neu gene of SEQ ID NO: 3 that lacks the transmembrane domain of Her-2/neu gene from the above plasmid, which results in the secretion of the expressed protein into the cell exterior.

Further, the inventive plasmid can be prepared by replacing the inherent signal sequence of Her-2/neu gene with exogenous signal sequence, such as the herpes simplex virus type I glycoprotein D signal (gDs) sequence which is known to facilitate the efficient expression and secretion of human immunodeficiency virus (HIV) type I gp160.

In a preferred embodiment of the present invention, $pNeu_{TM}$ and pCK_{TM} prepared by inserting the truncated Her-2/neu gene lacking the intracellular domain to pTV and pCK vectors, respectively; $pNeu_{ECD}$ and pCK_{ECD} prepared by deleting the transmembrane domain of Her-2/neu gene from $pNeu_{TM}$ and pCK_{TM}, respectively; and $pNeu_{TM}$-gDs and $pNeu_{ECD}$-gDs prepared by replacing the inherent Her-2/neu signal peptide sequence of $pNeu_{TM}$ and $pNeu_{ECD}$ with the signal sequence of glycoprotein D of herpes simplex virus type I.

The plasmids, $pNeu_{TM}$, pCK_{TM}, $pNeu_{ECD}$ and pCK_{ECD} have been deposited on June 26, 2002 with the Korean Culture Center of Microorganisms (KCCM) under the accession numbers KCCM-10393, KCCM-10396, KCCM-10394 and KCCM-10395 respectively.

Administration of the plasmids to BALB/c mouse induce Her-2/neu specific IgG antibody according to the signal peptide sequence in various aspects. Specifically, Her-2/neu specific IgG titer is very high in serum of the mouse administered with $pNeu_{TM}$ or $pNeu_{ECD}$, but it is relatively low in serum of the mouse administered with $pNeu_{TM}$-gDs or $pNeu_{ECD}$-gDs. On the other hand, all plasmids induce strong Her-2/neu-specific CTL response. Accordingly, a relative importance of Her-2/neu specific CTL and antibody was evaluated by using these plasmids to eliminate Her-2/neu-expressing cancer cell.

As a result, these plasmids induce complete protection against a small number of cancer cells, and anti-cancer effect of $pNeu_{ECD}$ and $pNeu_{ECD}$-gDs are not significantly different in a preventive and therapeutic model. However, when a large number of cancer cells are used in a therapeutic model, only $pNeu_{ECD}$ shows statistically significant anti-cancer effect. This supports that only strong CTL response is enough to prevent cancer, but both CTL and antibody are necessary to treat cancer.

Further, antitumor effect of a human Her-2/neu gene can be enhanced by the co-injection with cytokine gene, particularly, GM-CSF, IL-18 or IL-15, and the bicistronic plasmid expressing a human Her-2/neu gene and cytokine gene also showed preventive antitumor effects inducing the delay and inhibition of death caused by tumor metastasis suggesting that the plasmid is more effective in the clinical use.

Meanwhile, plasmids pCK_{TM} and pCK_{ECD} show almost similar effect to those of $pNeu_{TM}$ and $pNeu_{ECD}$, and this suggests that the inventive plasmid can be clinically used as a vaccine for treating cancer.

The composition of the present invention comprising the plasmid can be used as a therapeutic vaccine in reducing metastasis after tumor surgery and as a prophylactic vaccine for people with genetic high risk.

Typically, such vaccines are prepared as injectable, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to infection can also be prepared. The preparation can also be emulsified. The active immunogenic ingredients are often mixed with carriers which are pharmaceutically acceptable and compatible with the active ingredient. Suitable pharmaceutically acceptable carriers include, for example, one or more of saline, dextrose, glycerol, ethanol, or mixture thereof. In addition, if desired, the vaccine can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the vaccine.

Vaccine compositions of the present invention may be administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories, and in some cases, oral formulations. For suppositories,

traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5 to 10%, preferably 1 to 2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10% to 95% of active ingredient, preferably 25 to 70%.

The plasmid constructs of the present invention can be formulated into the vaccine compositions as neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with free amino groups of the peptide) and which are formed with inorganic acids such as hydrochloric or phosphoric acids, or with organic acids such as acetic, oxalic, tartaric, maleic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as sodium, potassium, ammonium, calcium or ferric hydroxides, and organic bases such as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

Vaccine are administered in a manner compatible with the dosage formulation. The quantity to be administered depends on the subject to be treated, including, e.g., capacity of the subject's immune system to synthesize antibodies, and the degree of protection or treatment depends on the degree of Her-2/neu of the subject. The inventive compounds as an active ingredient may be administered through oral route or parenteral route in an effective amount ranging from about 0.2 to 10 mg/kg (body weight), preferably from about 4 to 5 mg/kg (body weight) per day in a single dose or in divided doses in case of a mammal including a human being.

In one aspect, an adjuvant effect is achieved by use of an agent such as aluminum hydroxide or phosphate (aluminum) used in about 0.05 to about 0.1% solution in phosphate buffered saline, an admixture with synthetic polymers of sugars (Carbopol. R^{TM}) used as an about 0.25% solution and protein aggregation by heat treatment. Aggregation by reactivating with pepsin treated with antibodies to albumin (Fab), mixture with bacterial cell(s) such as

C. parvum or an endotoxin or a lipopolysaccharide component of Gram-negative bacteria, emulsion in physiologically acceptable oil vehicles such as mannide mono-oleate (Aracel A) or emulsion with a 20% solution of a perfluorocarbon (Fluosol-DA. R^{TM}) used as a block substitute also may be employed. Other examples of adjuvants include DDA (dimethyldioctadecylammonium bromide), QuilA, RIBI, monophosphoryl lipid A (MPL) and muramyl dipeptide (MDP) and so on may be used in the present invention. Other adjuvant such as aluminum hydroxide, aluminum phosphate (Adju-Phos), calcium phosphate, muramyl dipeptide analogue may be employed.

In addition, immunomodulators such as lymphokines (e.g., IFN-g, IL-2 and IL-12) or synthetic IFN-g inducers such as poly I:C can be used in combination with adjuvants described herein.

The reason for mixing the inventive plasmids with the adjuvants is to effectively activate the cellular immune reaction. However, this can be achieved by other way, such as effective antigen expression in vaccines of non-pathogenic microorganism. This microorganism includes BCG (*Mycobacterium bovis* BCG). As a non-pathogenic microorganism, bacteria such as *Mycobacterium*, *Salmonella*, *Pseudomonas*, *Escherichia*, and so on is preferred, and *Mycobacterium bovis* is more preferred.

The inventive living vaccines may be prepared by culturing the transformed non-pathogenic cells according to the present invention, displacing the cells to the medium for vaccines, and optionally adding carriers, vehicles and/or adjuvants.

The following Examples are intended to further illustrate the present invention without limiting its scope.

Reference Example 1: Cell lines and animals

The Her-2/neu expressing human breast carcinoma SK-BR3 cell line (ATCC HTB-30) and murine colon adenocarcinoma cell line CT26 (ATCC CRL-2639) were obtained from the American Type Culture Collection (Manassas, VA,

USA). Human breast cancer cell line SK-BR3 cells were maintained in RPMI1640 (BioWhittaker, Walkersvile, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO, Gaithersburg, MD) and 1% penicillin-streptomycin (GIBCO). Her-2/neu-expressing transfectoma Her2-CT26 cells were prepared by transduction of CT26 cells with the cDNA-encoding human Her-2/neu (NCBI: M11730). Her2/CT26 and CT26 cells were cultured in IMDM (BioWhittaker) containing 10% heat-inactivated fetal bovine serum (FBS, GIBCO, Gaithersburg, MD) and 1% penicillin-streptomycin (GIBCO).

Female 5-week-old BALB/C mice were purchased from Charles River (Osaka, Japan) and kept at 22℃, 55% relative humidity, and a daily lighting cycle of 12hrs light/ 12hrs dark with free access to food and water. The mice were kept in a germ-free isolator during the whole experiments.

Reference Example 2: Isolation of DNA plasmids for i.m. injection

Escherichia coli strain DH5 (Promega, Madison, WI, USA) transformed with each of the plasmids, $pNeu_{TM}$, pCK_{TM}, $pNeu_{ECD}$, pCK_{ECD}, $pNeu_{TM}$-gDs, $pNeu_{ECD}$-gDs and control vectors pTV2 and pCK, was grown in LB broth (Difco, Detroit, MI). Large-scale preparation of the plasmid DNA from the cultured transformant was carried out by using an Endofree Qiagen Plasmid-Giga kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. DNA was then precipitated, suspended in sterile PBS at a concentration of 2 ㎎/㎖, and stored in aliquots at -20℃ for subsequent use in immunization protocols.

Reference Example 3: Flow cytometry (FACS)

To examine whether antibody in serum could specifically reacts with Her-2/neu surface protein, SK-BR3, Her2-CT26 and CT26 cells were stripped from the culture flasks with a cell scraper (Nunc, Naperville, IL). Removed cells were washed in a buffer consisting of RPMI1640, 2% FBS and 0.1% sodium azide. Approximately 2×10^5 cells per analysis were incubated

together with a serial dilute of an antibody or control antibody at 4℃ for 30 min. Cells were washed with the buffer 3 times and then stained with an FITC-conjugated goat monoclonal antibody specific for mouse IgG (Sigma) at 4℃ for 30 minutes. The stained cells were washed with the buffer 2 times and resuspended with the buffer. To exclude dead cells from data, 1 ㎍/㎖ propidium iodide (Sigma) was added to the cell suspension and incubated for 5 minutes prior to analysis. Only the cells that were negative by propidium iodide staining were gated and further analyzed for binding to cancer cells. Flow cytometry was performed using a PAS IIIi flow cytometer (Partec GmbH, Münster, Germany).

Reference Example 4: Confocal microscopy for anti-Her-2/neu antibodies

Approximately 1×10^5 SK-BR3 cells were grown for three days on Lab-Tek chambered coverglass (Nunc, Naperville, IL) coated with 1 ㎎/㎖ poly-L-Lysine. The cells were fixed with 4% paraformaldehyde in PBS at room temperature for 10 min, washed three times with DMEM (BioWhittaker, Walkersvile, MD), blocked with DMEM containing 1% goat-globulin at 4℃ for 1 hour, incubated with 1:50 diluted mouse sera in a blocking solution at 4℃ for 8 hours, washed, and incubated with R-phycoerythrin-conjugated goat anti-mouse immunoglobulin secondary antibody (Southern Biotech, Birmingham, AL) at room temperature for 30 min. Slides were then mounted on Gel/Mount media (Fisher) and examined using a confocal microscopy (Leica TCS-SP laser scanning microscopy).

Reference Example 5: DNA immunization method

100 ㎍ of plasmid DNA that was dissolved in 100 ㎕ of sterile PBS was injected intramuscularly to two anterior tibialis of each mouse. The injection site was anesthetized with 50 ㎕ of bupivacaine-HCl (ASTRA, Westborough, MA). For daily immunization for therapeutic vaccination, bupivacaine-HCl was pretreated only once just before the first immunization. Sera were collected at selected time points and monitored for the presence of

anti-Her-2/neu antibodies.

Reference Example 6: Chromium-release assays

Splenocytes prepared by extracting spleen from immunized mice were cultured with mytomycin C (Sigma) treated Her2-CT26 cells for 6 days. Her2-CT26 or CT26 tumor target cells were labeled with ^{51}Cr by incubating 2×10^6 cells with 200 μCi $Na^{51}CrO_4$ in 200 ㎕ saline at 37℃ for 90 min. The unincorporated ^{51}Cr was removed by washing with RPMI1640 four times. After 6 days, the serially diluted splenocytes suspended in RPMI supplemented with 10% FBS were mixed with 10,000 labeled target cells in the wells of a round-bottom microtiter plate. The plate was incubated at 37℃ for 4 hours. After the incubation, a 100 ㎕ aliquot was removed from each well for counting with a scintillation counter (Packard, Minaxi Auto Gamma 5000 Series). The percent lysis was calculated by formula 1:

<Formula 1>

Specific lysis (%) = $100 \times [(cpm_{experimental} - cpm_{spontaneous}) / (cpm_{max} - cpm_{spontaneous})]$

The cpm_{max} value was determined by adding 10 ㎕ of 5% triton-X (Sigma) to the wells containing ^{51}Cr-labeled target cells. Each group was tested in duplicate. The $cpm_{spontaneous}$ value was determined by adding only an equal volume of the medium without the addition of splenocytes or triton-X.

Reference Example 7: Tumor challenge

Mice were challenged by injection with Her2-CT26 cells suspended in sterile PBS either subcutaneously on the flank or intravenously. The three-dimensional size of each tumor was measured with a caliper, and the volume was calculated by formula 2:

<Formula 2>

tumor volume (㎣) = (width × length × depth) ㎥ × $(1/2)^3$ × 4π/3

Animals were palpated twice a week for the development of tumors. Mice showing any symptom of acute sickness, hard to breathe or rare movement were sacrificed.

Example 1: Construction of Her-2/neu expressing plasmids

The cDNA encoding the entire human Her-2/neu gene (SEQ ID NO: 1) was inserted into *Hin*dIII/*Xba*I site of the pRC/CMV (Invitrogen Life technologies) to produce a full-length Her-2/neu plasmid (9.6 Kb).

The plasmid $pNeu_{ECD}$ and pCK_{ECD} comprising the extracellular domain of Her-2/neu without the intracellular and transmembrane domains of Her-2/neu, was generated from the PCR product of the obtained full-length Her-2/neu plasmid using NF6 (SEQ ID NO: 4) and NSR1 (SEQ ID NO: 5) as a primer pair, and cloned into the *Kpn*I and *Xba*I sites of pTV2 (Lee, S. W., et al., *J. Virol.*, 72:8430-8436, 1998) and pCK (Lee, Y., et al., *Biochem. Biophys. Res. Commun.*, 272:230-235, 2000; Accession No.: KCCM-10179), respectively. PCR was carried out at 94℃ for 2 min; 25 cycles of 94℃ for 15 sec, 55℃ for 30 sec and 68℃ for 3.5 min; and 72℃ for 7 min.

Further, in order to obtain plasmid $pNeu_{TM}$ and pCK_{TM} encoding the extracellular and transmembrane domains of Her-2/neu without the intracellular domain of Her-2/neu, the PCR was repeated except for using NF5 SEQ ID NO: 6) and NRM2 (SEQ ID NO: 7) as a primer pair, and the PCR product was cloned into the *Kpn*I and *Xba*I sites of pTV2 and pCK, respectively (Fig. 1a).

Then, in order to obtain the plasmid $pNeu_{ECD}$-gDs, encoding the extracellular domain of Her-2/neu without the intracellular and transmembrane domains of Her-2/neu and the herpes simplex virus type I glycoprotein D (gD) signal sequence instead of Her-2/neu signal sequence, PCR was repeated except for using NSF2 (SEQ ID NO: 8) and NSR1 (SEQ ID NO: 5) as a primer pair, and the PCR product was cloned into the *Asc*I and *Xba*I sites of pTV2-gDs (gD signal sequence was cloned into the *Pst*I and *Asc*I sites of pTV2 vector). Further, in order to obtain the plasmid $pNeu_{TM}$-gDs, encoding the extracellular

and transmembrane domains of Her-2/neu and the gD signal sequence instead of Her-2/neu signal sequence, PCR was repeated except for using NF3 (SEQ ID NO: 9) and NRM2 (SEQ ID NO: 7) as a primer pair, and cloned into the *Asc*I and *Xba*I sites of pTV2-gDs (Fig. 1a).

Example 2: Induction of Her-2/neu specific antibody by Her-2/neu DNA vaccination

Whether various pNeu plasmid constructs prepared in Example 1 could induce Her-2/neu specific antibodies were examined as follows. 100 μg of plasmid DNA was injected intramuscularly to each mouse prepared in Reference Example 1 three times according to a preset immunization schedule (Fig. 1b). Some mice of each group were sacrificed to obtain spleen, and Her-2/neu-specific CTL was determined. Other mice were challenged with Her-2/neu expressing cancer cell to evaluate anti-cancer immunity.

Sera were obtained from mice before the first injection and one week after the third injection. The Her-2/neu specific antibody titer in the serum was measured based on the binding of the antibody to the breast cancer cell line, SK-BR3, using a flow cytometry as described in Reference Example 3. Her-2/neu specific antibody titers of all mice vaccinated with $pNeu_{TM}$, $pNeu_{TM}$-gDs, $pNeu_{ECD}$ or $pNeu_{ECD}$-gDs were determined, and the greatest dilution of serum for which a shift in the mean fluorescence intensity by the binding to SK-BR3 cells, compared to that of control antibody, was shown in Table 1.

<Table 1>

pTV2 (n=5)	$pNeu_{TM}$ (n=5)	$pNeu_{ECD}$ (n=5)	$pNeu_{TM}$-gDs (n=5)	$pNeu_{ECD}$-gDs (n=5)
<50	12800	12800	800	<50
<50	12800	12800	50	<50
<50	3200	12800	<50	<50
<50	12800	12800	800	<50
<50	3200	12800	50	<50

As shown in Table 1, the observed Her-2/neu specific antibody titers

were ranked in the order of $pNeu_{ECD}$ > $pNeu_{TM}$ > $pNeu_{TM}$-gDs > $pNeu_{ECD}$-gDs = pTV2. As expected, none of the sera collected from animals before the injection of plasmid DNA had detectable Her-2/neu specific binding activities. Moreover, none of the animals injected with control vector pTV2 made detectable anti-Her-2/neu antibodies at 1:50 dilution (Fig. 2a). However, vaccination with $pNeu_{TM}$ or $pNeu_{ECD}$ resulted in high Her-2/neu specific IgG titers, and serum samples diluted by 1:800 revealed a wide shift in the mean fluorescence intensity (Figs. 2b and 2c). In contrast, vaccination with $pNeu_{TM}$-gDs or $pNeu_{ECD}$-gDs resulted in a very low IgG titer, and serum samples diluted by 1:50 revealed a little or a barely detectable shift in the mean fluorescence intensity (Figs. 2d and 2e). Serum samples obtained from immunized mice 10 days after the third injection with plasmid pCK_{ECD} and pCK_{TM}, respectively and diluted by 1:400 revealed a wide shift in the mean fluorescence intensity. Therefore, it is confirmed that the plasmids induce Her-2/neu specific antibody (Fig. 3).

Further, the existence of Her-2/neu-specific antibodies in mouse sera immunized with $pNeu_{TM}$ or $pNeu_{ECD}$-gDs was also confirmed by confocal microscopic analysis as described in Reference Example 4. Mouse serum immunized with $pNeu_{TM}$ (Fig. 4b) demonstrated clear localization of Her-2/neu specific antibodies bound on the surface of SK-BR3, compared with those of control vector pTV2 (Fig. 4a) or $pNeu_{ECD}$-gDs (Fig. 4c), which is consistent with the Her-2/neu specific antibody titers presented in Fig. 2.

Example 3: Induction of Her-2/neu specific CTL by Her-2/neu DNA vaccination

Since Her-2/neu-specific antibody responses in vaccinated mice were fluctuated with type of pNeu constructs used in immunization as described in Example 2, Her-2/neu-specific CTL responses induced in the same mice were evaluated as follows.

Splenocytes were obtained 2 weeks after the third immunization from the same mice that were tested for Her-2/neu specific antibody titers in sera. Splenocytes were cultured with mytomycin-C-treated human Her-2/neu

expressing syngeneic murine transfectoma, Her2-CT26 cells for 6 days, and were assayed for the lysis of CT26 or Her2-CT26 target cells by chromium release assay for 4 hours.

As a result, splenocytes from mice vaccinated with $pNeu_{TM}$ (Fig. 5b), $pNeu_{ECD}$ (Fig. 5c), $pNeu_{TM}$-gDs (Fig. 5d) or $pNeu_{ECD}$-gDs (Fig. 5e) exhibited CTL-dependent lysis of Her-2/CT26, compared with splenocytes from control vector pTV2 vaccinated control mice (Fig. 5a), and the relative strength of Her-2/neu specific CTL response was in order of $pNeu_{TM}$ > $pNeu_{ECD}$ > $pNeu_{TM}$-gDs > $pNeu_{ECD}$-gDs $\gg$ pTV2. Her-2/neu specific lysis by splenocytes from mice immunized with any one of pNeu constructs was comparable to the others of pNeu constructs and were 80~90% at an E:T (effector:target) ratio of 50:1 and 60~70% at an E:T ratio of 10:1 (Figs 5b to 5e). However, splenocytes from any group of mice did not induce CTL-dependent lysis of CT26 cells.

Her-2/neu specific CTL response was assayed in case of pCK_{ECD} and pCK_{TM} prepared by replacing $pNeu_{ECD}$ and $pNeu_{TM}$ to pCK. As shown in Fig. 6, pCK_{ECD} and pCK_{TM} plasmids induced CTL responses comparable to those of $pNeu_{ECD}$ and $pNeu_{TM}$, and the plasmid comprising both extracellular domain (ECD) and transmembrane domain induced higher CTL responses than those of the plasmid comprising only ECD.

In brief, all Her-2/neu expressing plasmids induced strong Her-2/neu specific CTL response, which was irrelevant to their signal peptide sequences and vectors. However, they induced significantly different Her-2/neu specific antibody responses according to their signal peptide sequences, and this result was confirmed by repeated tests.

Example 4: Preventive antitumor effect by Her-2/neu DNA vaccine

Antitumor immunity against Her-2/neu expressing syngeneic murine tumor cell line Her2-CT26 in BALB/c mouse was evaluated as follows. Initially, titration studies were performed to determine the optimal number of tumor cells to be injected s.c. or i.v. into mice to generate subcutaneous tumor formation or lung metastasis. As a result, Her2-CT26 cells induced subcutaneous or lung metastatic tumor when 5×10^4 cells or more were

injected s.c. or i.v.. Since a long survival period may help to distinguish antitumor efficacy of Her-2/neu DNA plasmids, 5×10^4 cells were chosen as the initial cell number for i.v. or s.c. tumor challenge. Each mouse received three i.m. injections of 100 μg plasmid DNA according to a preset immunization schedule (Fig. 1b) and 10 days after the third injection of plasmid DNA, each mouse was challenged i.v. or s.c. with 5×10^4 Her2-CT26 cells.

As a result, in the above subcutaneous tumor model study, all of the animals injected with control vector, pTV2, developed palpable tumors (Fig. 7a). On the other hand, tumors were completely suppressed in all groups of mice each injected with $pNeu_{TM}$, $pNeu_{TM}$-gDs, $pNeu_{ECD}$ or $pNeu_{ECD}$-gDs for 60 days following s.c. tumor injection. In a metastasis model, all group of mice injected with $pNeu_{TM}$, $pNeu_{TM}$-gDs, $pNeu_{ECD}$ or $pNeu_{ECD}$-gDs survived i.v. tumor challenge (Fig. 7b). However, four of the seven mice (57%) injected with only pTV2 and all mice injected with only PBS did not survive lung metastasis.

Further, to determine the antitumor effect in case of pCK_{ECD} and pCK_{TM} prepared by replacing $pNeu_{ECD}$ and $pNeu_{TM}$ to pCK, mice were vaccinated intramuscularly three times with 100 μg plasmid DNA. The mice were challenged s.c. or i.v. with 1×10^6 Her2-CT26 cells 10 days after final vaccination. As a result, in case of the s.c. injection with Her-2/CT26, solid tumors were grew all mice both injected with PBS and control pCK vector. However, five of the eight mice (62.5%) and seven of the eight mice (87.5%) injected with pCK_{ECD} and pCK_{TM} showed no tumors suggesting that the plasmids significantly inhibited tumor growth (Fig. 8a). In case of i.v. injection, all mice both injected with PBS and pCK vector died of tumor metastasis within 17 days, but the survival rate of the mice both injected with pCK_{ECD} and pCK_{TM} was significantly increased (Fig. 8b). Accordingly, it is confirmed that the plasmid having Her-2/neu gene in the pCK vector has excellent antitumor effect as well as pNeu plasmid having Her-2/neu gene in the pTV2 vector.

<u>Example 5</u>: Comparison of antitumor immunity by $pNeu_{ECD}$ and $pNeu_{ECD}$-gDs

The number of tumor cells to be injected was increased by a factor of 100 (5×10^6/mouse) for s.c. tumor challenge and by a factor of 40 (2×10^6/mouse) for i.v. tumor challenge. It was impossible to use a cell number of more than 2×10^6 for i.v. tumor challenge because there was the danger of blood vessel blockage by excessive tumor cells injected i.v.. For comparison, a set of $pNeu_{ECD}$ and $pNeu_{ECD}$-gDs that generated the largest difference in Her-2/neu-specific antibody titers among the four different Her-2/neu-expressing plasmids was selected.

Each mouse received three i.m. injections of 100 μg plasmid DNA according to the same immunization schedule (Fig. 1b), and 10 days after the third injection of plasmid DNA, each mouse was challenged s.c. with 5×10^6 or i.v. with 2×10^6 Her-2/CT26. In the subcutaneous model, all eight animals injected with pTV2 developed tumors and the mean tumor volume reached over 2000 mm^3 before day 19 post s.c. tumor challenge. The mean tumor volume of eight mice injected with $pNeu_{ECD}$ was 82.2 mm^3 at day 23 and that of eight mice injected with $pNeu_{ECD}$-gDs was 67.9 cm^3. While there was significant tumor growth suppression in mice injected with $pNeu_{ECD}$ ($p = 2.9900e-8$, Student's *t* test) or $pNeu_{ECD}$-gDs ($p= 2.8400e-8$, Student's *t* test), the difference in the mean tumor volume between the two immunized groups was not statistical significant ($P =0.8684$, Student's *t* test). In the metastasis model, lung metastasis was inhibited until day 40 in eight of the eight mice (100%) injected with $pNeu_{ECD}$ and in seven of the eight mice (88%) injected with $pNeu_{ECD}$-gDs. All mice injected with pTV2 did not survived lung metastasis. In other words, although the survival was significantly prolonged by treatment with $pNeu_{ECD}$ ($p < 0.0001$, Mantel-Haenszel test) or $pNeu_{ECD}$-gDs ($p < 0.0001$, Mantel-Haenszel test) compared with pTV2, there was no significant difference between $pNeu_{ECD}$ and $pNeu_{ECD}$-gDs ($p = 0.3173$, Mantel-Haenszel test) (Figs. 9a and 9b).

Example 6: Efficacy of Her-2/neu DNA vaccine in a therapeutic model

To compare the antitumor immunity efficacies of $pNeu_{ECD}$ and $pNeu_{ECD}$-

gDs in a therapeutic model, mice were challenged with tumor cells first, and then injected with DNA plasmids. 6-week old naive mice were challenged i.v. with 1×10^5 or 5×10^5 Her2-CT26 cells, and then were divided into 4 groups. 1 hour after the tumor injection, each mouse received the first i.m. injection of 100 μg of $pNeu_{ECD}$ or $pNeu_{ECD}$-gDs, followed by four more daily i.m injections with the same DNA plasmid.

When 1×10^5 tumor cells were injected, all mice treated with $pNeu_{ECD}$ or $pNeu_{ECD}$-gDs survived lung metastasis for the following 40 days (Fig. 10a). However, five of the eight mice (63%) injected with only pTV2 and eight of the eight mice (100%) injected with only PBS did not survive lung metastasis. Although $pNeu_{ECD}$ and $pNeu_{ECD}$-gDs improved the survival rate significantly ($p=0.0085$, Mantel-Haenszel test) as compared with pTV2, there was no significant difference between $pNeu_{ECD}$ and $pNeu_{ECD}$-gDs.

On the other hand, when the number of tumor cells was increased 5 times (5×10^5), only the mice injected with $pNeu_{ECD}$ exhibited an increased survival rate which was statistically significantly ($p = 0.0237$, Mantel-Haenszel test, Fig. 10b) compared with mice injected with pTV2. However, the mice injected with $pNeu_{ECD}$-gDs did not show significantly enhanced survival ($p = 0.4628$, Mantel-Haenszel test) as compared with the mice injected with pTV2. Nonetheless, consistently with the preventive model, there was no significant difference in antitumor immunity between $pNeu_{ECD}$ and $pNeu_{ECD}$-gDs ($p = 0.4263$, Mantel-Haenszel test).

Further, to determine the therapeutic effects of pCK_{ECD} and pCK_{TM} prepared by replacing $pNeu_{ECD}$ and $pNeu_{TM}$ to pCK, mice were challenged with i.v. with 2×10^5 Her2-CT26 cells, and then injected with DNA plasmids. In the groups injected with pCK_{TM} and pCK_{ECD} plasmids, all mice showed significantly prolonged survival rate, while in the groups injected with PBS and pCK, all mice died of lung metastasis of cancer cells within 21 days (Fig. 11). Accordingly, it is confirmed that the plasmid having Her-2/neu gene in the pCK vector has excellent antitumor therapeutic effect as well as pNeu plasmid having Her-2/neu gene in the pTV2 vector.

Example 7: Construction of cytokine plasmids

As a result of Examples 2 to 6, it is confirmed that injection of DNA vaccine lacking the intracellular domain of Her-2/neu into a mouse showed antitumor effects by producing antigen specific antibodies and cytotoxic T lymphocytes. All subsequent Her-2/neu DNA vaccine experiments were performed by using pCK_{TM} which has excellent antitumor effect. In order to improve the antitumor effect of Her-2/neu DNA vaccine, a method co-injecting cytokines were deviced. The co-injection of a cytokine improves the delivery efficiency into antigen presenting cells (APC) and induce Th1 type immune response so that it is more effective to remove cancer cells in the clinical use and helpful for overcoming immune tolerance against Her-2/neu in tumor patients. The present invention chose Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) and FMS-like tyrosine kinase 3 ligand (Flt3L) which improve the delivery efficiency into antigen presenting cells (APC), and Early T lymphocyte activation-1 (Eta-1), Interleukin-12 (IL-12), IL-15 and IL-18 which are typical T_H1 skewing cytokines expected to induce immune responses. Then the 6 cytokine genes were inserted into PCK vector to obtain a vector expressing cytokine. GM-CSF is related to the growth and differentiation of dendritic cell, macrophage, monocyte or granulocyte cell, Eta-1 is a cytokine important to cell-mediated immune responses, and Flt3L is related to the growth and differentiation of stem cells, particularly, important to the differentiation of dendritic cells. IL-12, IL-15 and IL-18 belong to interleukin family are also important to cell-mediated immune responses, and is a representative cell-mediated immune responses-related cytokine.

Eta-1 (SEQ ID NO: 10), IL-18 (SEQ ID NO: 11), IL-15 (SEQ ID NO: 12) and Flt3L (SEQ ID NO: 13) genes were amplified from mRNA isolated from the spleen of BALB/c mice by using reverse transcriptase (SUPERSCRIPT™ II RT, GIBCO BRL) with specific primers (Eta-1, with EF1 of SEQ ID NO: 14 and ER1 of SEQ ID NO: 15; IL-18, with 18F1 of SEQ ID NO: 16 and 18R1 of SEQ ID NO: 17; IL-15, with 15F1 of SEQ ID NO: 18 and 15R1 of SEQ ID NO: 19; and Flt3L, with FF1 of SEQ ID NO: 20 and FR1 of SEQ ID NO: 21) to obtain PCR products. The PCR products were inserted

into the *Kpn*I-*Xba*I and *Eco*RI-*Xba*I sites of pCK to generate pCK-Eta1, pCK-IL18, pCK-IL15 and pCK-Flt3L. pCK-GMCSF and pCK-IL12 were constructed by inserting the *Eco*RI-*Xba*I and *Xho*I fragments of pTV2-GMCSF (Cho, J. H. et al., *Vaccine* 17: 1136-1144, 1999) and pTV2-IL12 (Ha, S. J. et al., *Nat. Biotechnol.* 20: 381-386, 2002), into pCK vector, respectively.

Example 8: Immune responses and antitumor activities induced by co-injection of Her-2/neu DNA and cytokine genes

To analyze immune responses and antitumor activities induced by co-injection of Her-2/neu DNA and cytokine genes, BALB/c mice were co-injected with pCK_{TM} and each of cytokine expressing vector of Example 7, and immune responses induced by the co-injection was compared with that induced by the single injection of pCK_{TM}. As shown in Fig. 12a, 100 μg of pCK_{TM} and 100 μg of each of cytokine expressing vectors were intramuscularly injected every 3 weeks totally 2 times. 3 weeks after the final vaccination, Her-2/neu specific antibody response was measured, and CTL responses was measured by using Her2-CT26 cells.

As shown in Table 2, production of Her-2/neu-specific antibodies were similar with that of pCK_{TM}.

<Table 2>

Mice	Her-2/neu specific IgG titer							
	PCK	pCK_{TM}	+IL-12	+IL-15	+IL-18	+Eta-1	+Flt3L	+GM-CSF
1	<50	3200	6400	6400	6400	3200	800	3200
2	<50	3200	3200	3200	6400	3200	3200	6400
3	<50	6400	6400	1600	6400	6400	800	6400
4	<50	3200	400	1600	12800	6400	1600	1600

Further, Table 3 shows a summary of the CTL responses observed in Fig. 12b. As shown in Table 3, the Her-2/neu specific CTL response induced by vaccination of pCK_{TM} with Eta-1 or Flt3L was increased as compared with that induced by pCK_{TM} only, but that induced by vaccination of pCK_{TM} with IL-

18, GMCSF or IL-15 was decreased. The CTL responses were increased or decreased according to the type of co-injected cytokine, but it is universally higher than that of single injection of pCK vector.

<Table 3>

	Effector : Target ratio		
	3:1	10:1	30:1
pCK	5.5±0.69	8.8±3.16	20.3±5.43
pCK_{TM}	52.6±6.03	67.6±0.56	76.4±1.21
pCK_{TM} + pCK-IL12	34.6±5.67	59.3±8.82	79.3±6.99
pCK_{TM} + pCK-IL15	39.0±0.76	53.6±1.08	70.7±6.30
pCK_{TM} + pCK-IL18	21.8±1.44	38.8±4.53	47.7±2.55
pCK_{TM} + pCK-Eta1	59.5±9.01	88.7±11.07	96.2±4.52
pCK_{TM} + pCK-Flt3L	48.4±2.99	79.6±3.22	95.9±2.38
pCK_{TM} + pCK-GMCSF	34.6±12.96	50.6±15.56	64.3±13.8

To determine antitumor activity induced by co-injection of Her-2/neu DNA and a cytokine gene, BALB/c mice were co-injected i.m. with 100 μg pCK_{TM} and 100 μg each of the pCK-cytokine plasmids every 3 weeks, totally 2 times to induce immune response, and challenged i.v. or s.c. with 1×10^6 Her2-CT26 at week 3 after the 2^{nd} vaccination (Fig. 13a). As a result, the preventive antitumor activity for the growth of subcutaneous tumor was improved with co-vaccination with pCK_{TM} and a cytokine, as compared with the inhibition effect of pCK_{TM}. Especially, the inhibition effect by pCK-GMCSF, pCK-IL15 and pCK-Eta1 were the best, then the effect by pCK-Flt3L and pCK-IL12 were good (Fig. 13b). In the preventive antitumor effect for metastatic tumor, metastases of intravenously challenged Her2-CT26 were fully inhibited by vaccination with in the order of pCK-GMCSF > pCK-IL12, but the effect by other cytokines were similar with that of pCK_{TM} (Figs. 13c and 13d).

In order to investigate the therapeutic effect for cancer induced by co-injection of pCK_{TM} and each of the cytokine plasmids, Mice were vaccinated every 2 days, totally 3 times after 2×10^5 Her2-CT26 i.v. challenge (Fig. 14a).

As a result, most co-vaccination with pCK_{TM} and pCK-cytokine plasmids, in particular, IL-15, IL-18 and Flt3L showed better survival rate than vaccination with only pCK_{TM} (Figs. 14b and 14c). Therefore, the antitumor activity of pCK_{TM} may be promoted by co-injection of a particular cytokine plasmid such as pCK-GMCSF, pCK-IL18 and pCK-IL15.

Example 9: Construction of bicistronic plasmids expressing Her-2/neu and cytokine

In the study using cytokine genes as an adjuvant of DNA vaccines, one plasmid vector containing an antigen and the other plasmid vector containing a cytokine may be co-injected. However, recently, it is reported that a DNA vaccine is prepared in the form of a bicistronic plasmid expressing an antigen and a cytokine at the same time, and its excellent effect (Barouch, D. H., et al., *J. Immunol.* 168:562-568, 2002). Co-expression of the antigen and the cytokine in a vector can be achieved by using two promoters in the vector, or using IRES sequence derived from HCV virus (Ha, S. J. et al., *Nat. Biotechnol.* 20: 381-386, 2002). In the present invention, Her-2/neu gene was inserted next to the CMV promoter, IRES was connected thereto, and the cytokines gene was inserted thereto to simultaneously express antibody together with cytokine (Fig. 15a).

To generate bicistronic plasmids co-expressing Her-2/neu and cytokine proteins, the *Bam*HI-*Nco*I fragment of IRES having the nucleotide sequence of SEQ ID NO: 22 was separated from pCK-IL12, inserted into the *Bam*HI-*Nco*I site of pCK_{TM}, and each of PCR products of GM-CSF, Flt3L, IL-15, IL-18 and Eta-1 genes prepared in Example 7 were inserted into the *Nco*I -*Not*I site of pCK_{TM}. For IL-12 and IL-23 (Belladonna, M. L., et al., *J. Immunol.* 168: 5448-5454, 2002), IRES was amplified by PCR using pCK-IL12 as a template and IRES-F1 of SEQ ID NO: 23 and IRES-R1 of SEQ ID NO: 24 as a primer pair, and the amplified product was inserted into the *Not*I-*Xho*I site of pCK_{TM}, and IL-12 or IL-13 was inserted into the *Xho*I-*Xba*I site of pCK_{TM}.

Example 10: Antitumor effects induced by bicistronic plasmids expressing Her-2/neu and cytokine

To evaluate antitumor effects induced by bicistronic plasmids expressing Her-2/neu and cytokine, 100 μg of the antibody-cytokine vector prepared in Example 9 was intramuscularly injected every 3 weeks totally 2 times to induce the immune response, and then 1×10^6 Her2-CT26 cancer cells were injected i.v. or s.c. (Fig. 13a).

As a result, all Her-2/neu-cytokine vectors, specifically, pCK_{TM}-IL12, pCK_{TM}-Flt3L and pCK_{TM}-GMCSF, showed excellent preventive antitumor effects inhibiting the growth of solid tumor (Fig. 15b). For antitumor effects inducing the delay or inhibition of death caused by tumor metastasis, pCK_{TM}-Flt3L showed slightly lower inhibition effects for death caused by early metastatic cancer than pCK_{TM}, but other cytokines showed similar effects with pCK_{TM} (Figs 15c and 15d).

Further, to evaluate the anti-metastatic effect, 2×10^5 Her2-CT26 cancer cells were injected i.v. to induce tumor metastasis, and then 100 μg of each DNA vaccines were injected every 3 weeks totally 2 times (Fig. 14a). As a result, all DNA vaccines showed anti-metastatic effect as compared with pCK, and vaccination with pCK_{TM}-GMCSF and pCK_{TM}-IL18 prolonged the survival rate than pCK_{TM} (Figs. 16a and 16b). Accordingly, vaccination with bicistronic pCK_{TM}-cytokine vectors, specifically, pCK_{TM}-GMCSF and pCK_{TM}-IL18, showed excellent antitumor effects. Therefore, it is confirmed that the co-injection of human Her-2/neu gene and cytokine gene is more effective in the clinical application.

Effect of the Invention

The Her-2/neu DNA vaccine of the present invention can be effectively used as a therapeutic vaccine in reducing metastasis after tumor surgery or as a prophylactic vaccine for people with genetic high risk.

What is claimed is:

1. A plasmid construct having anti-cancer activity which is prepared by inserting a truncated human Her-2/neu gene lacking the intracellular domain, which has the nucleotide sequence of SEQ ID NO: 2, into plasmid pTV2 or pCK.

2. The plasmid construct of claim 1, wherein the truncated human Her-2/neu gene further lacks the transmembrane domain, and has the nucleotide of SEQ ID NO: 3.

3. The plasmid construct of claim 2, wherein the signal peptide of the human Her-2/neu gene is replaced by the signal peptide sequence of herpes simplex type I glycoprotein D (gD).

4. The plasmid construct of any of claims 1 to 3, which is selected from the group consisting of $pNeu_{TM}$ (KCCM-10393), $pNeu_{ECD}$ (KCCM-10394), pCK_{TM} (KCCM-10396), pCK_{ECD} (KCCM-10395), $pNeu_{TM}$-gDs and $pNeu_{ECD}$-gDs.

5. The plasmid construct of claim 1, which co-expresses both a Her-2/neu gene and a cytokine gene.

6. The plasmid construct of claim 5, wherein the cytokine gene is selected from the group consisting of granulocyte-macrophage colony-stimulating factor (GM-CSF), FMS-like tyrosine kinase 3 ligand (Flt3L), early T lymphocyte activation-1 (Eta-1), interleukin-12 (IL-12), IL-15 and IL-18.

7. A composition for preventing and/or treating cancer, which comprises the plasmid construct of claim 1 as an active ingredient.

8. The composition of claim 7, which further comprises a plasmid expressing cytokine gene.

9. The composition of claim 8, wherein the cytokine gene is selected from the group consisting of GM-CSF, Flt3L, Eta-1, IL-12, IL-15 and IL-18.

10. A DNA vaccine for preventing and/or treating cancer, which comprises the plasmid construct of claim 1 as an active ingredient.

11. The DNA vaccine of claim 10, which further comprises a plasmid expressing cytokine gene.

12. The DNA vaccine of claim 11, wherein the cytokine gene is selected from the group consisting of GM-CSF, Flt3L, Eta-1, IL-12, IL-15 and IL-18.

FIGURE

Fig. 1a

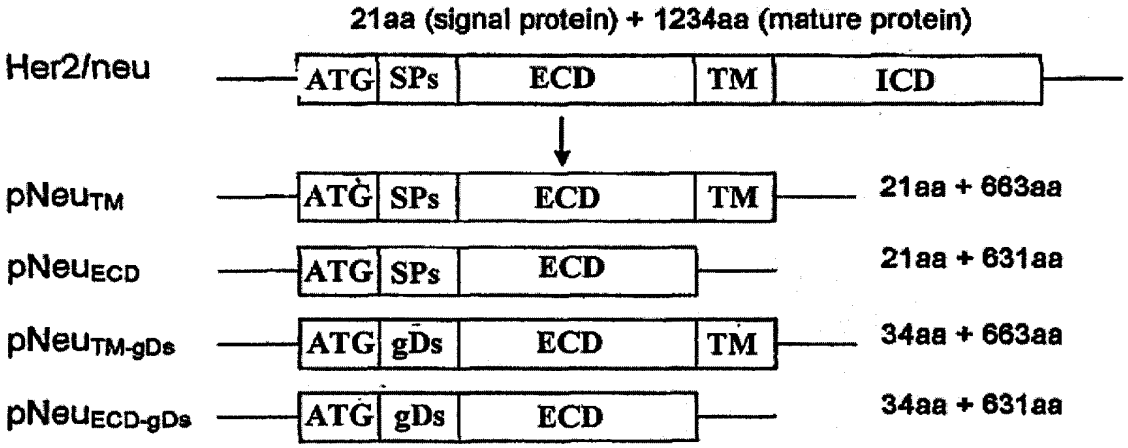


Fig. 1b

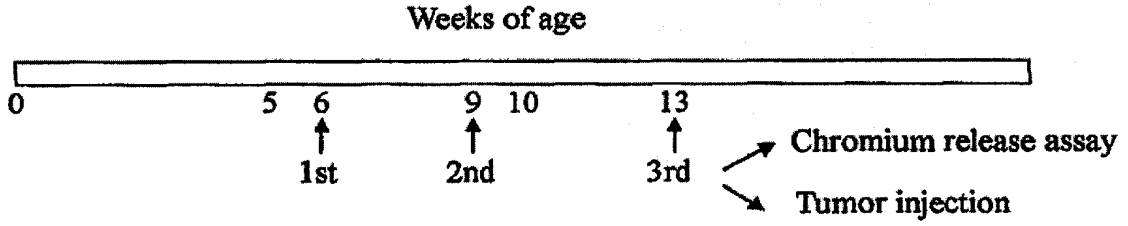


Fig. 2

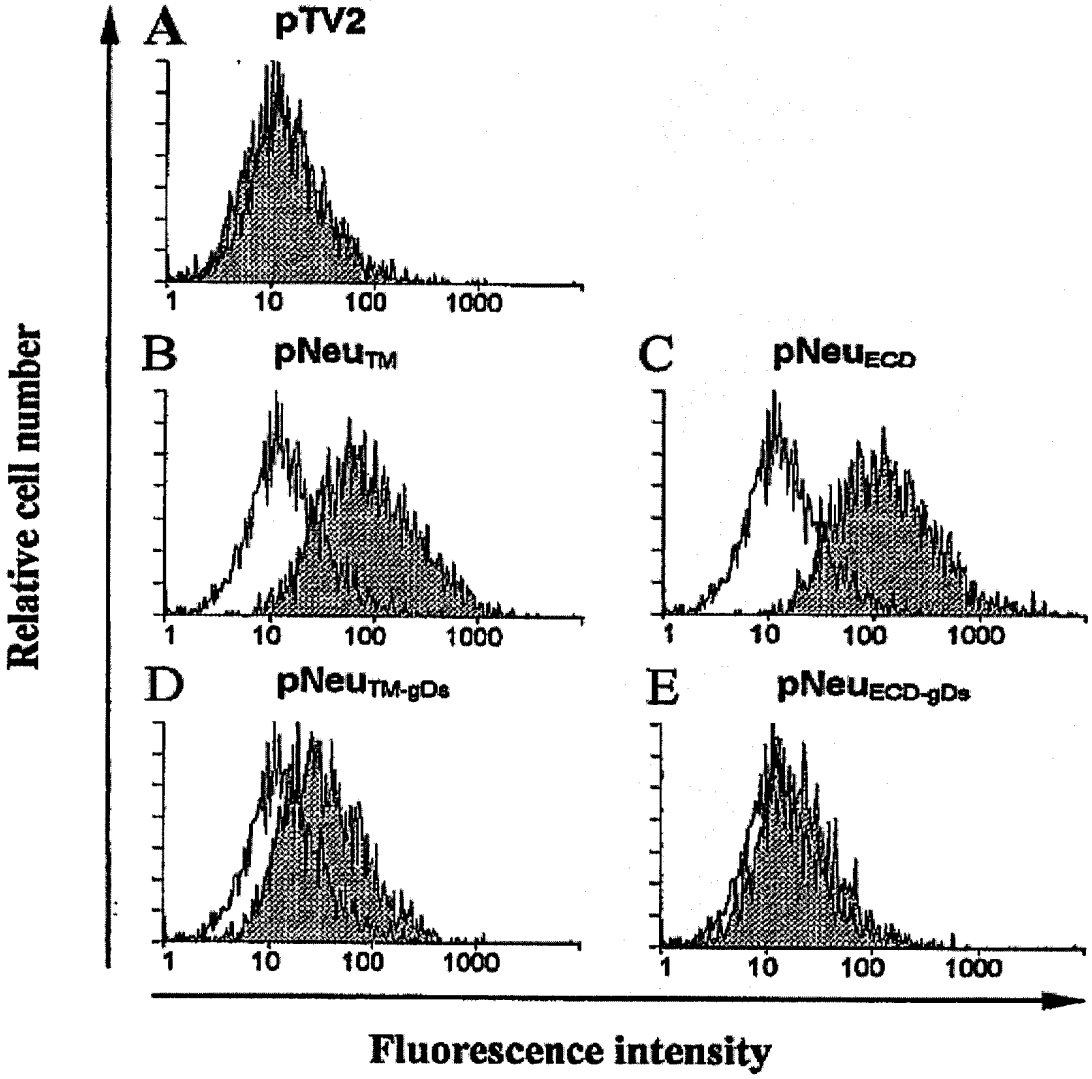

A
pTV2
B
pNeu$_{TM}$
C
pNeu$_{ECD}$
D
pNeu$_{TM-gDs}$
E
pNeu$_{ECD-gDs}$
1
10
100
1000
Relative cell number
Fluorescence intensity

Fig. 3

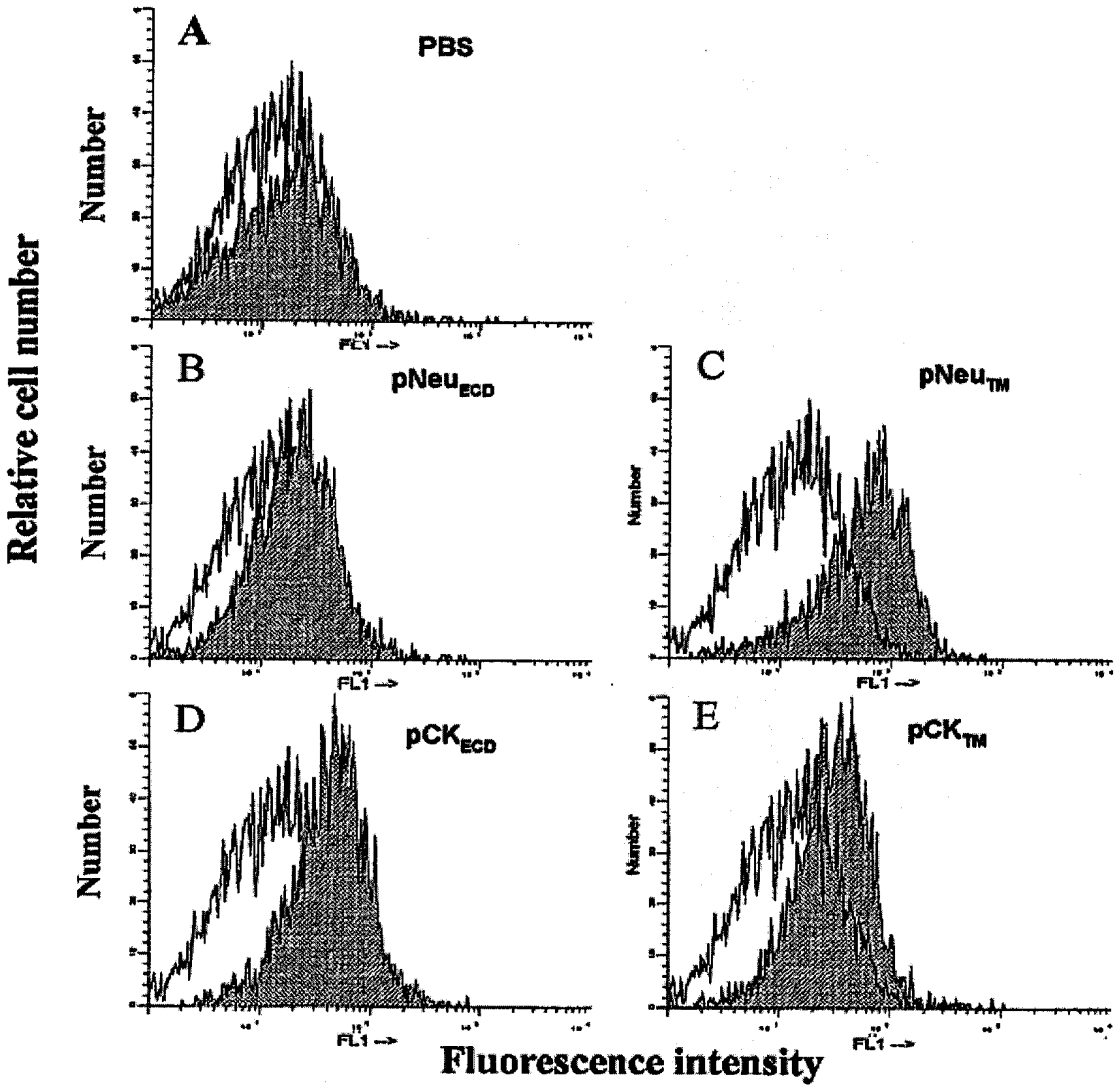

A
PBS
B
pNeu$_{ECD}$
C
pNeu$_{TM}$
D
pCK$_{ECD}$
E
pCK$_{TM}$
Number
Relative cell number
FL1 →
Fluorescence intensity

Fig. 4

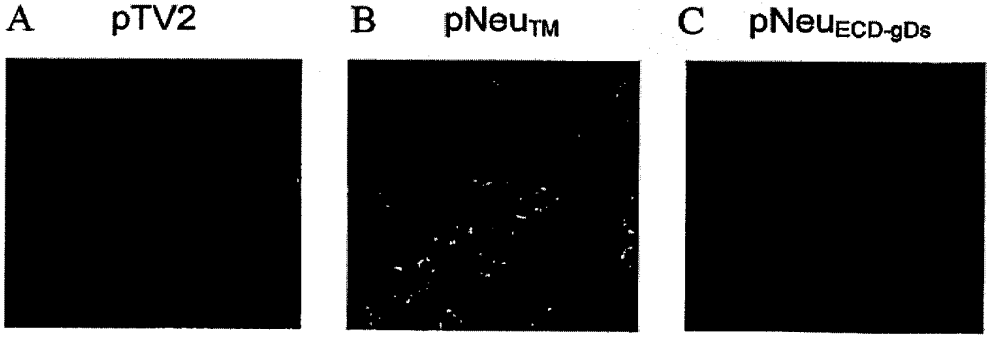

A pTV2
B pNeu$_{TM}$
C pNeu$_{ECD-gDs}$

Fig. 5

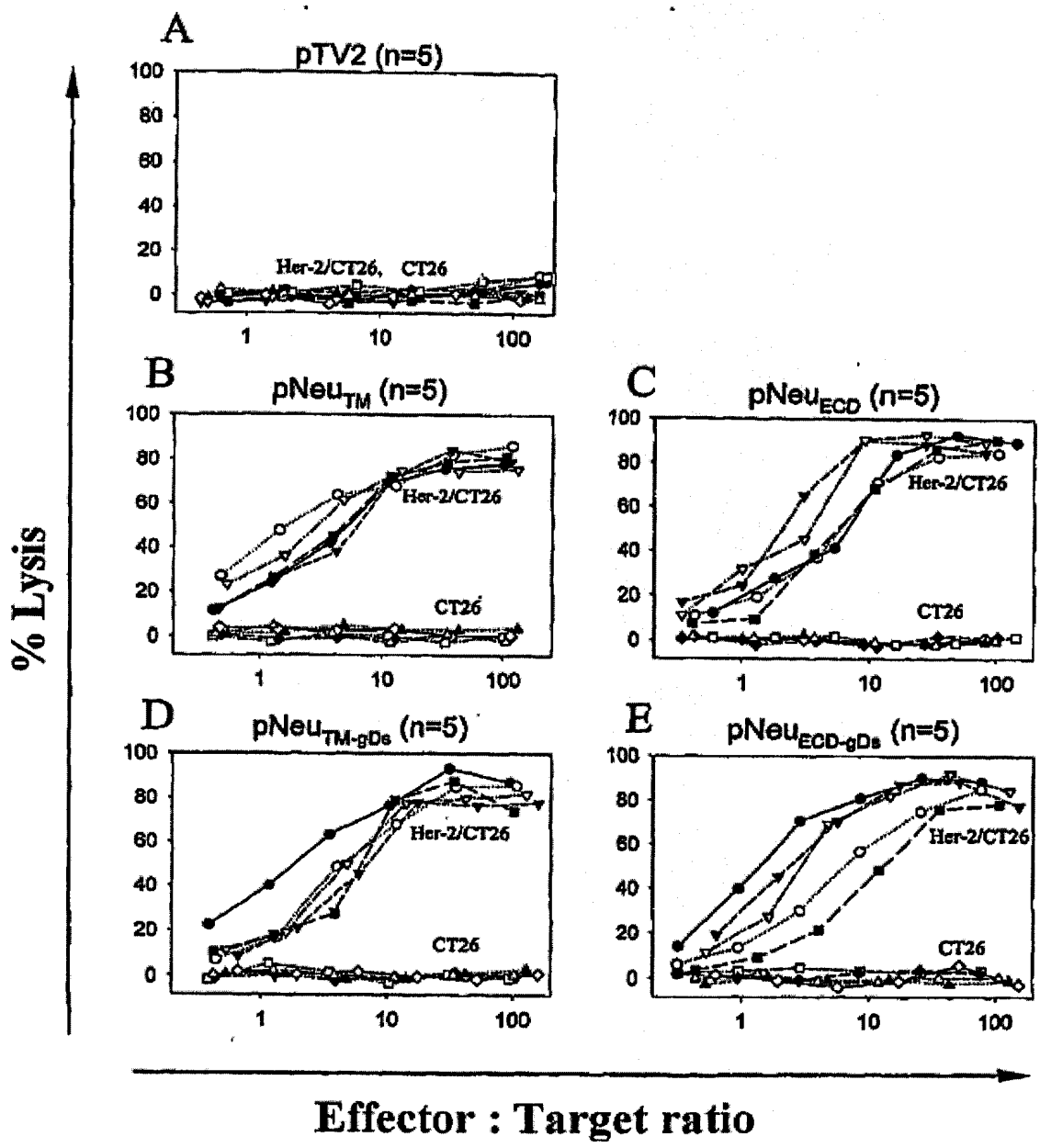

A
pTV2 (n=5)
B
pNeu$_{TM}$ (n=5)
C
pNeu$_{ECD}$ (n=5)
D
pNeu$_{TM-gDs}$ (n=5)
E
pNeu$_{ECD-gDs}$ (n=5)
Her-2/CT26, CT26
Her-2/CT26
CT26
% Lysis
Effector : Target ratio

Fig. 6

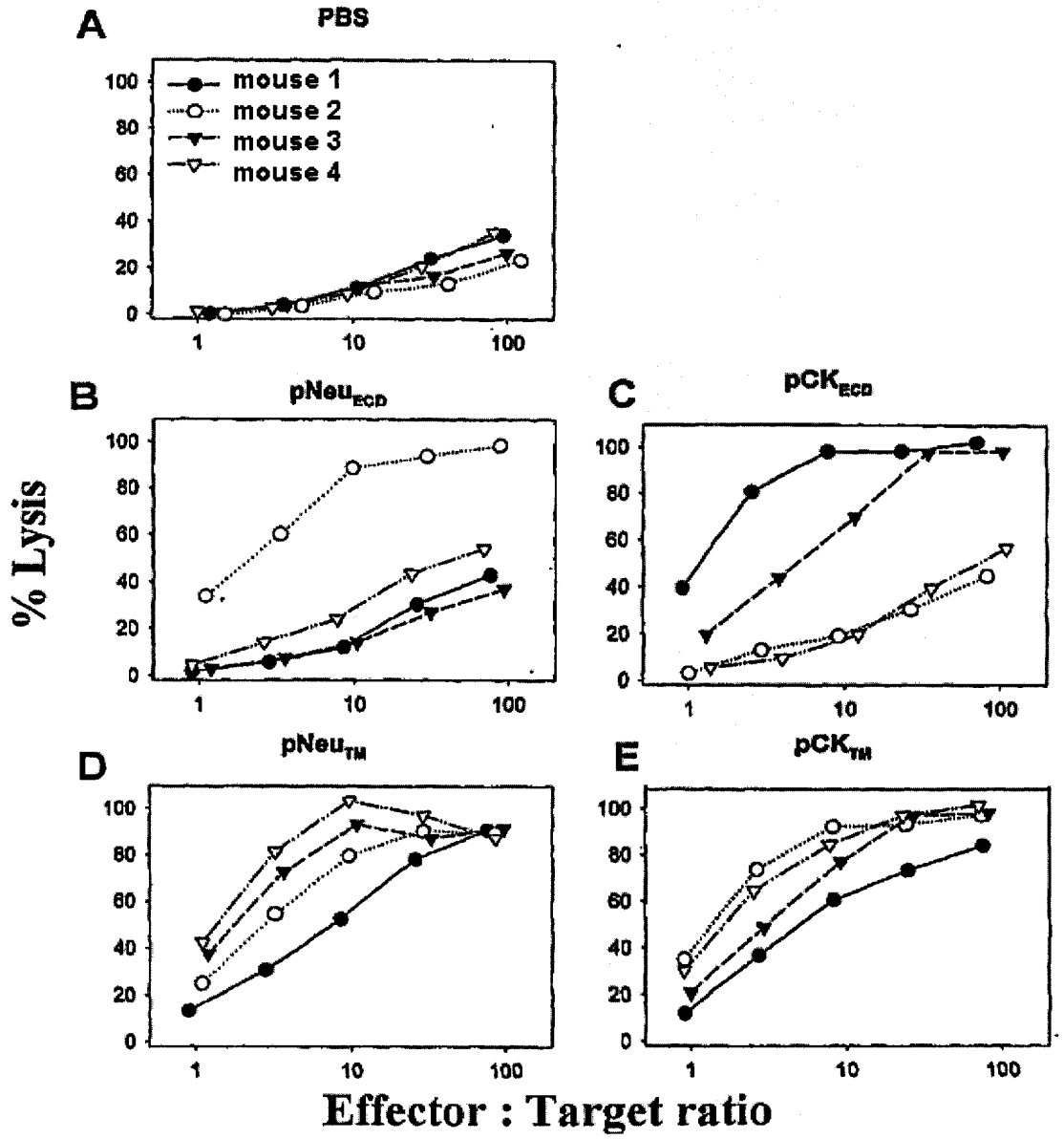

A
PBS
mouse 1
mouse 2
mouse 3
mouse 4
B
pNeu$_{ECD}$
C
pCK$_{ECD}$
D
pNeu$_{TM}$
E
pCK$_{TM}$
% Lysis
Effector : Target ratio

Fig. 7a

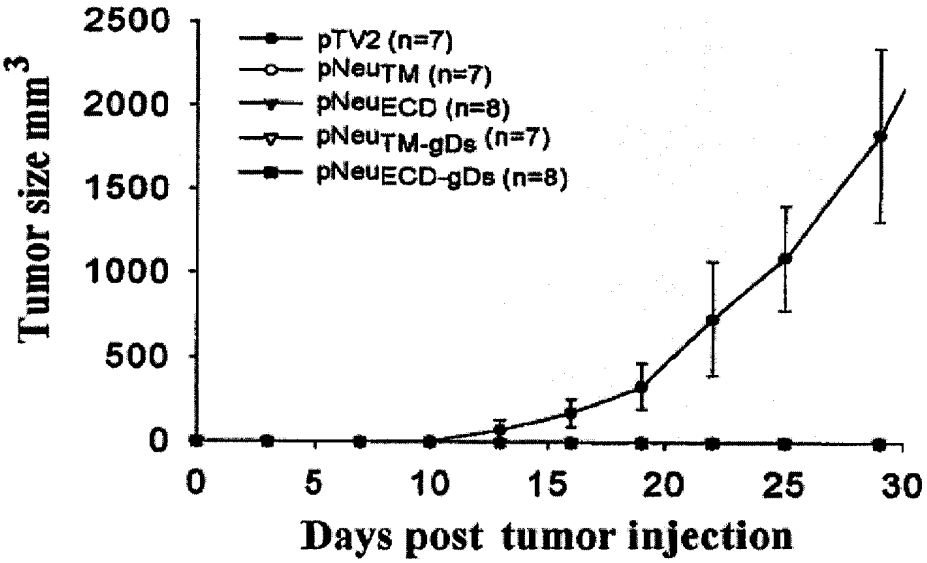

Tumor size mm3
2500
2000
1500
1000
500
0
pTV2 (n=7)
pNeuTM (n=7)
pNeuECD (n=8)
pNeuTM-gDs (n=7)
pNeuECD-gDs (n=8)
0
5
10
15
20
25
30
Days post tumor injection

Fig. 7b

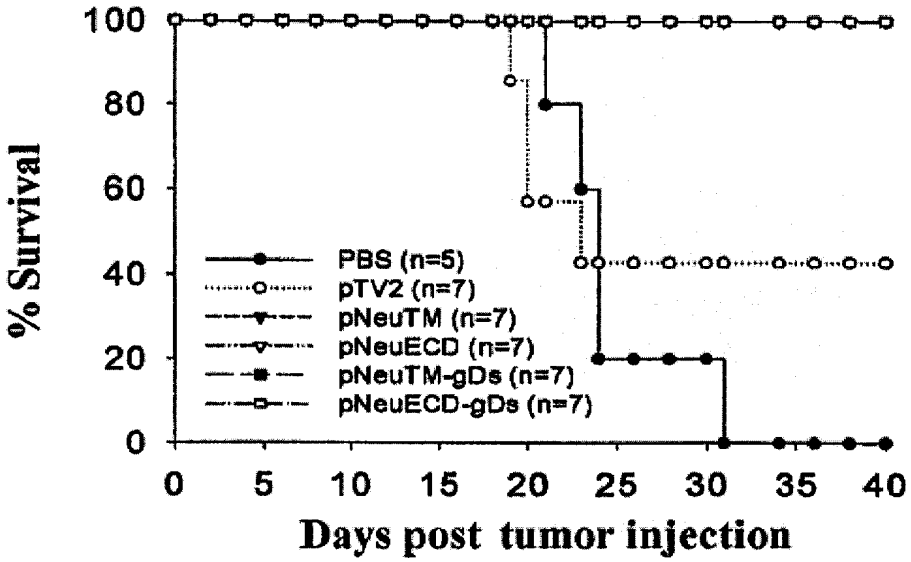

% Survival
100
80
60
40
20
0
PBS (n=5)
pTV2 (n=7)
pNeuTM (n=7)
pNeuECD (n=7)
pNeuTM-gDs (n=7)
pNeuECD-gDs (n=7)
0
5
10
15
20
25
30
35
40
Days post tumor injection

Fig. 8a

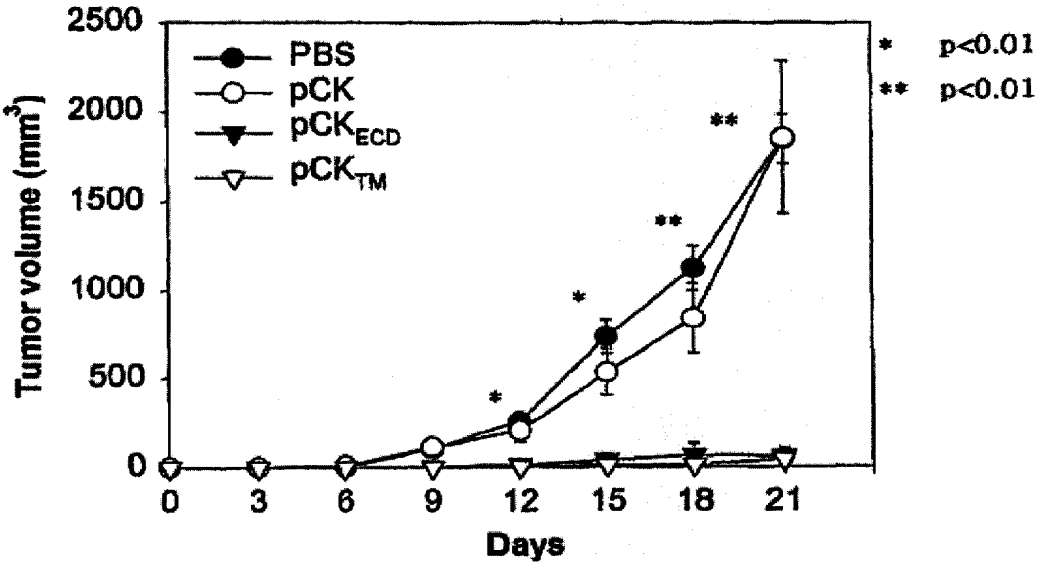

2500
2000
1500
1000
500
0
Tumor volume (mm³)
PBS
pCK
pCK$_{ECD}$
pCK$_{TM}$
*
**
**
*
0
3
6
9
12
15
18
21
Days
* p<0.01
** p<0.01

Fig. 8b

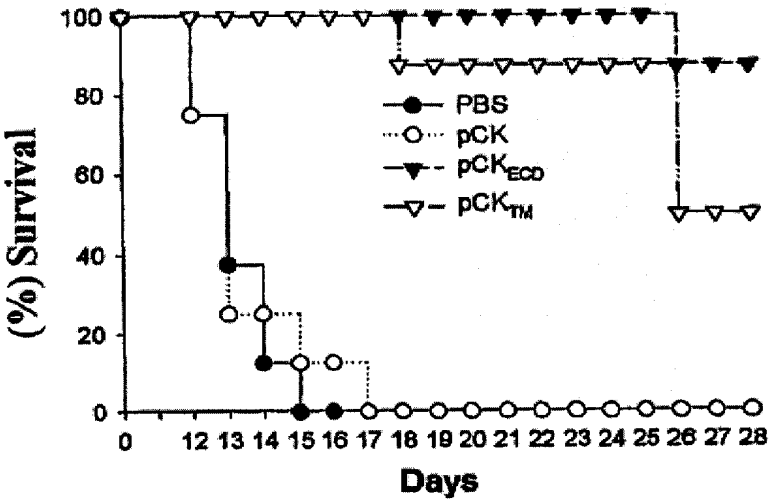

100
80
60
40
20
0
(%) Survival
PBS
pCK
pCK$_{ECD}$
pCK$_{TM}$
0
12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
Days

Fig. 9a

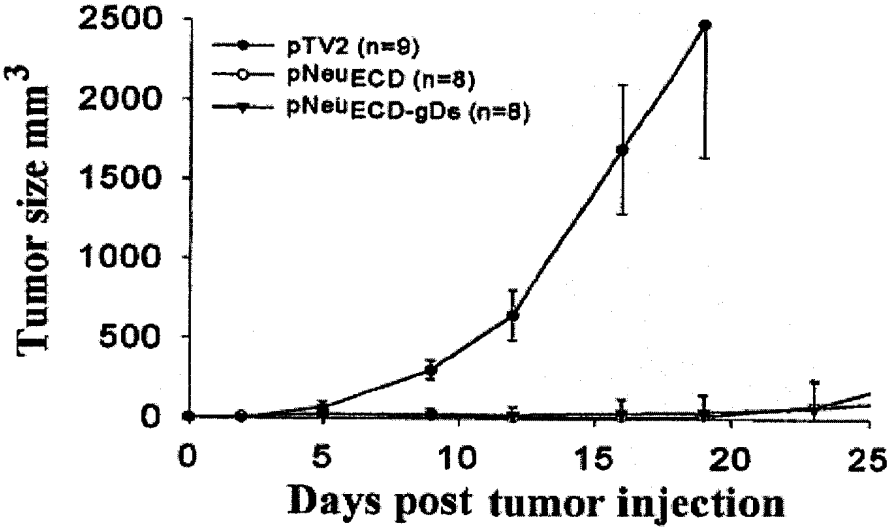

Tumor size mm3
2500
2000
1500
1000
500
0
pTV2 (n=9)
pNeuECD (n=8)
pNeuECD-gDs (n=8)
0
5
10
15
20
25
Days post tumor injection

Fig. 9b

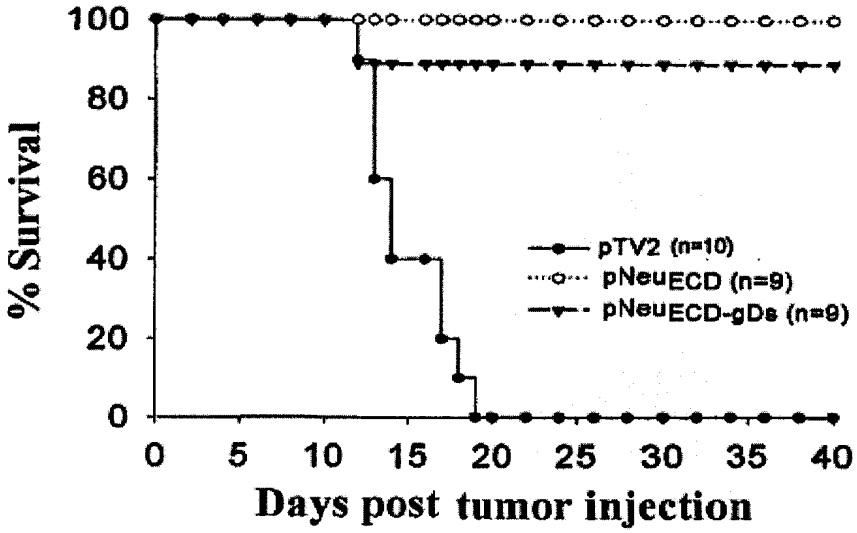

% Survival
100
80
60
40
20
0
pTV2 (n=10)
pNeuECD (n=9)
pNeuECD-gDs (n=9)
0
5
10
15
20
25
30
35
40
Days post tumor injection

Fig. 10a

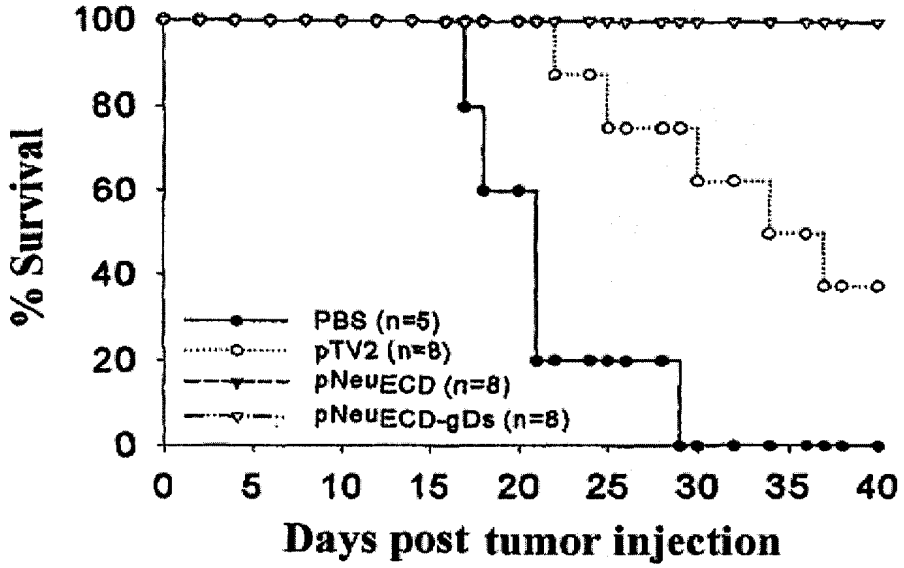


Fig. 10b

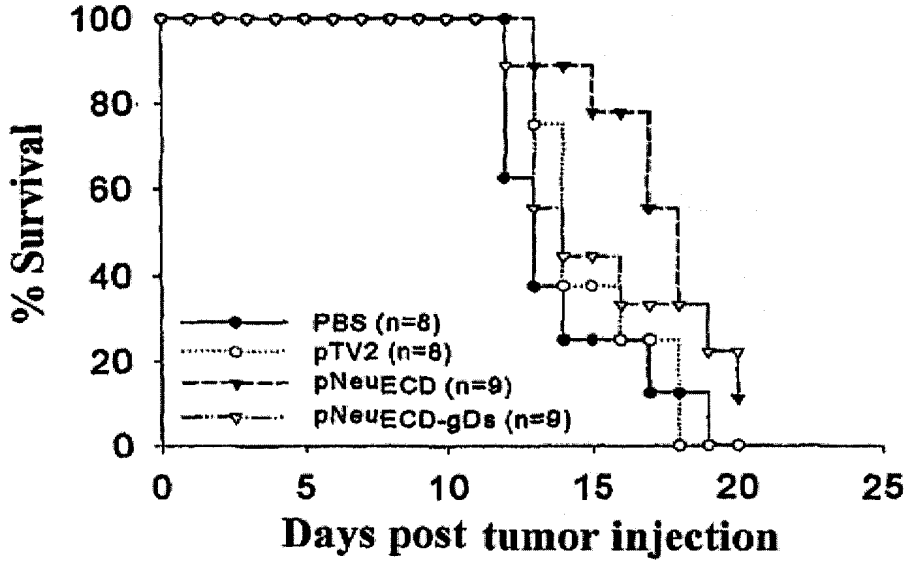


Fig. 11

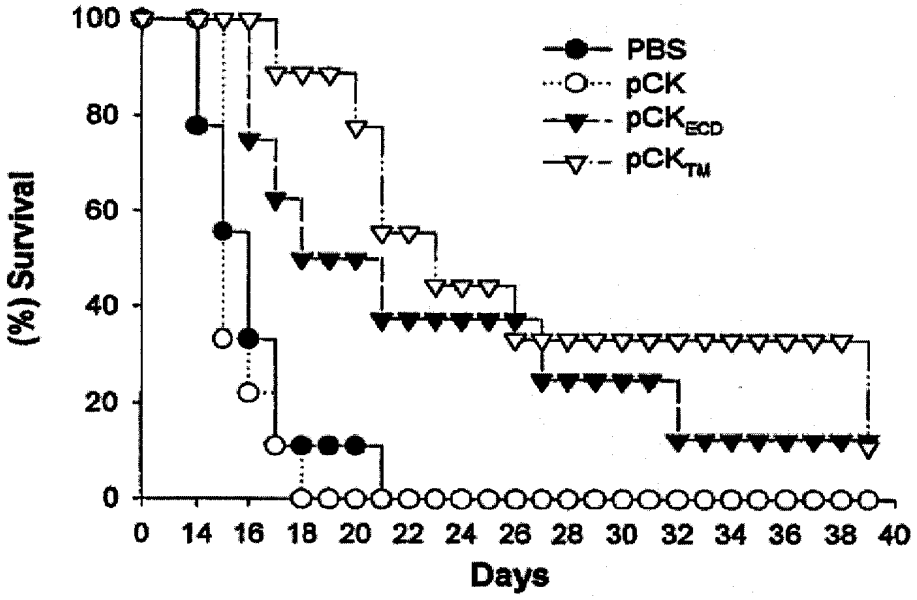


Fig. 12a

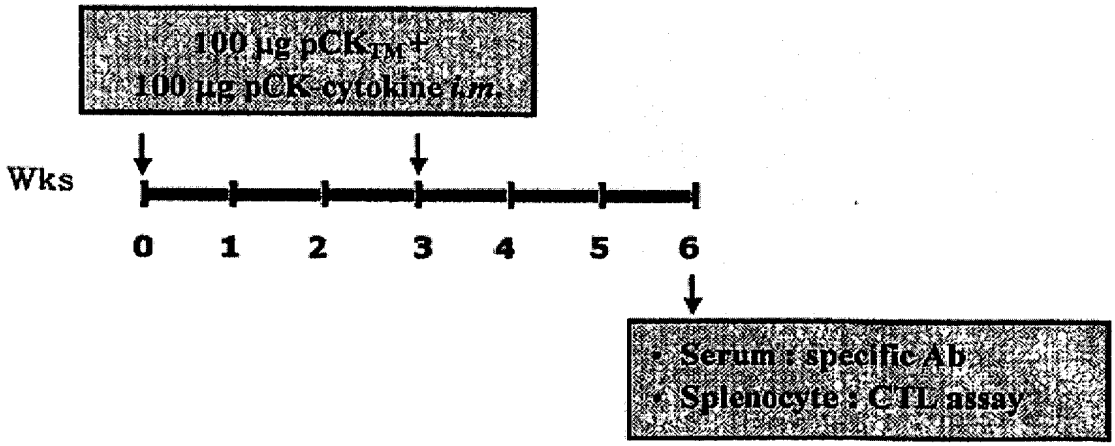


Fig. 12b

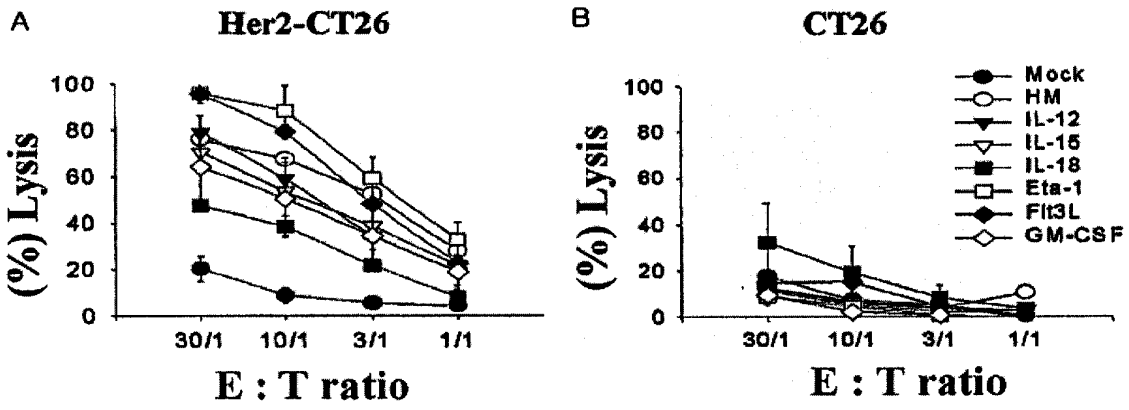


Fig. 13a

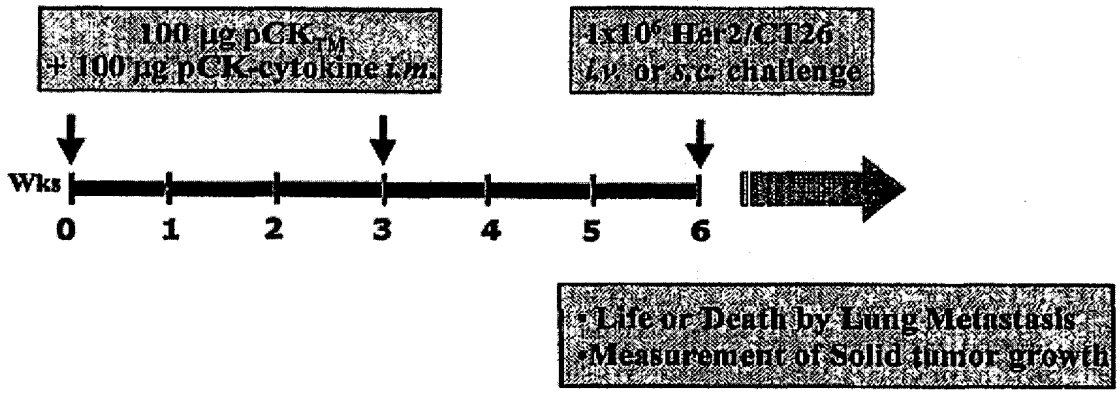


Fig. 13b

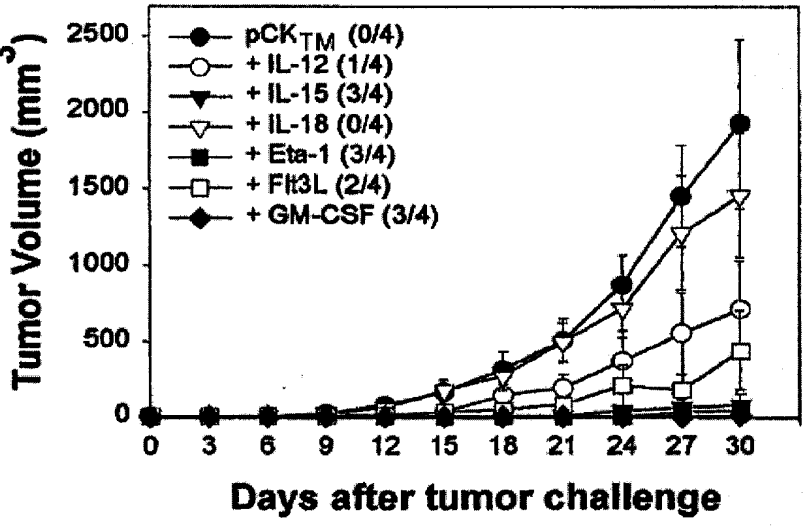


Fig. 13c

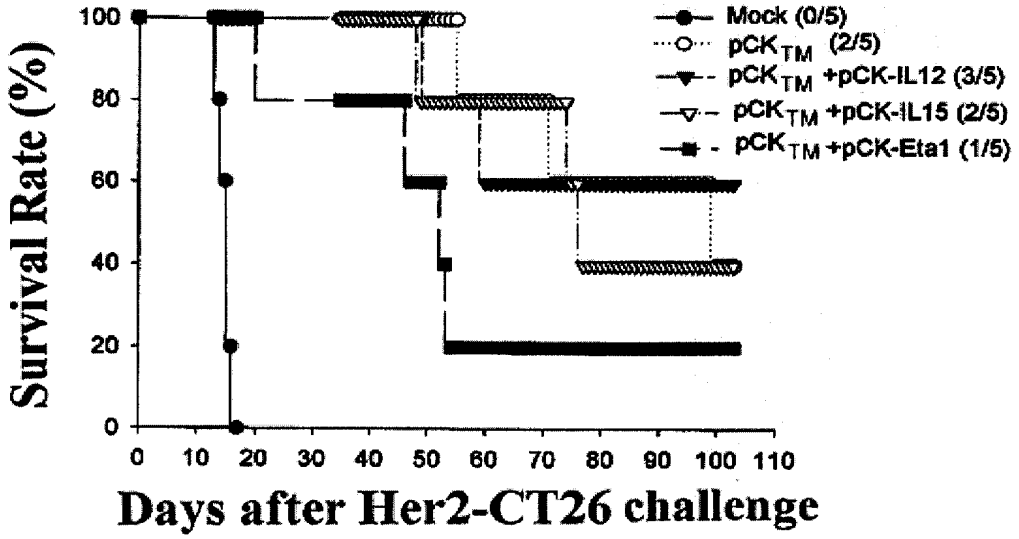


Fig. 13d

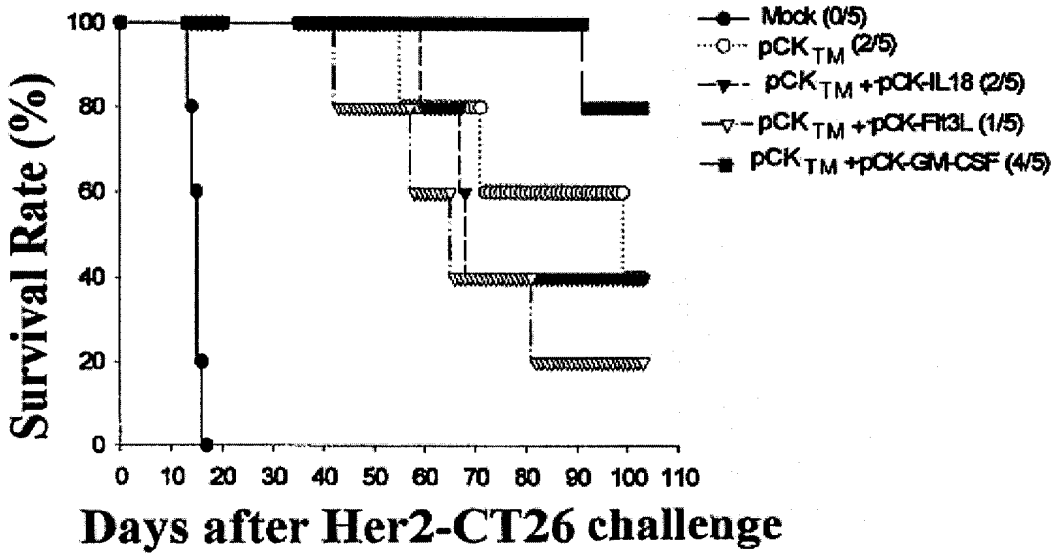


Fig. 14a

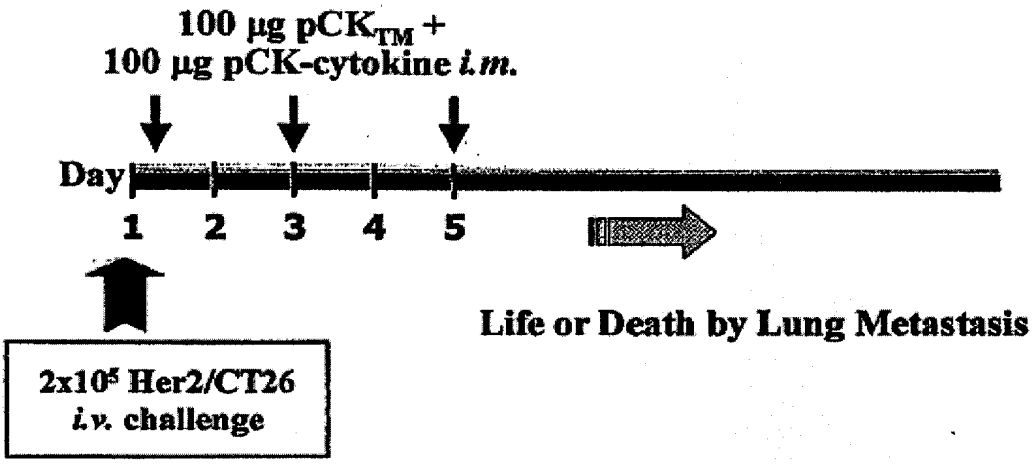


Fig. 14b

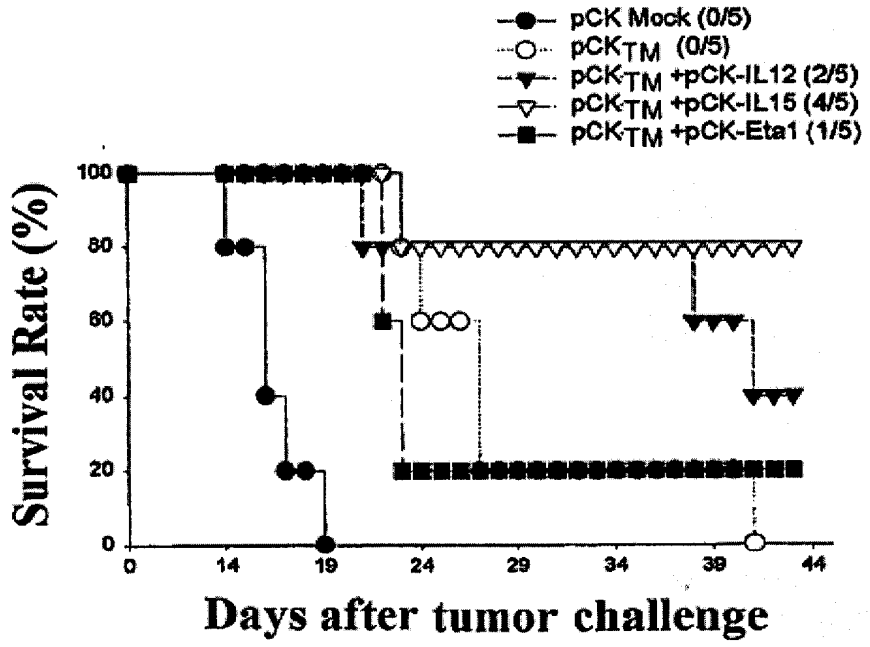


Fig. 14c

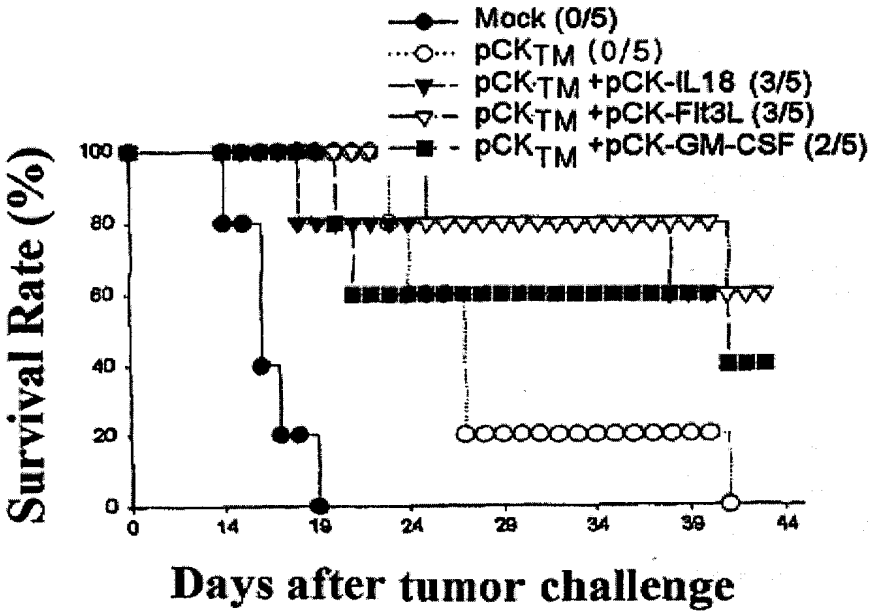


Fig. 15a

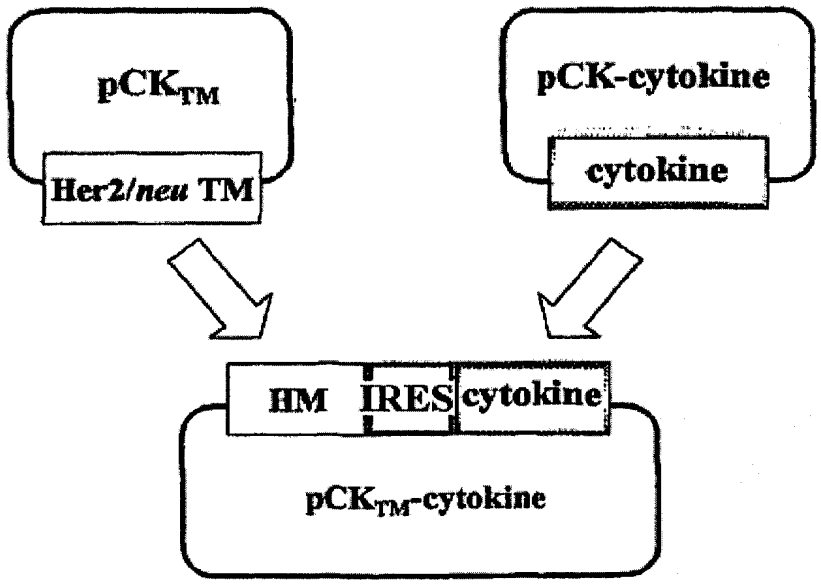


Fig. 15b

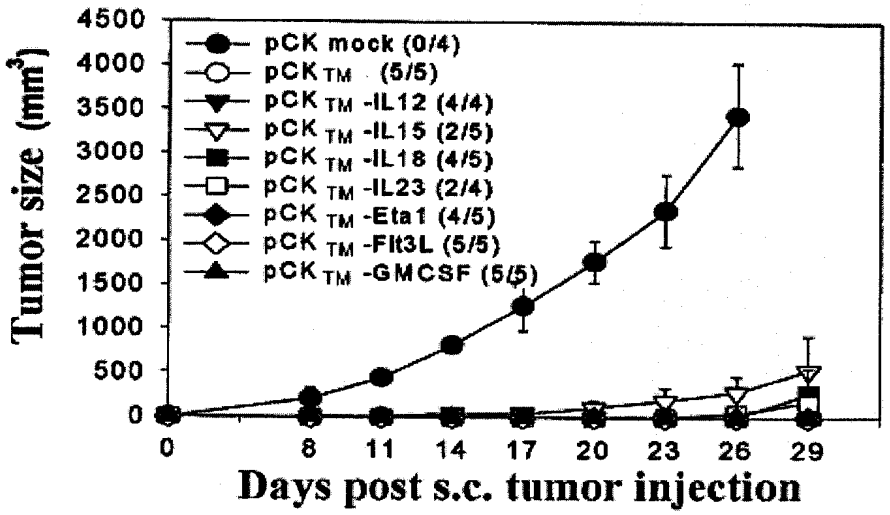


Fig. 15c

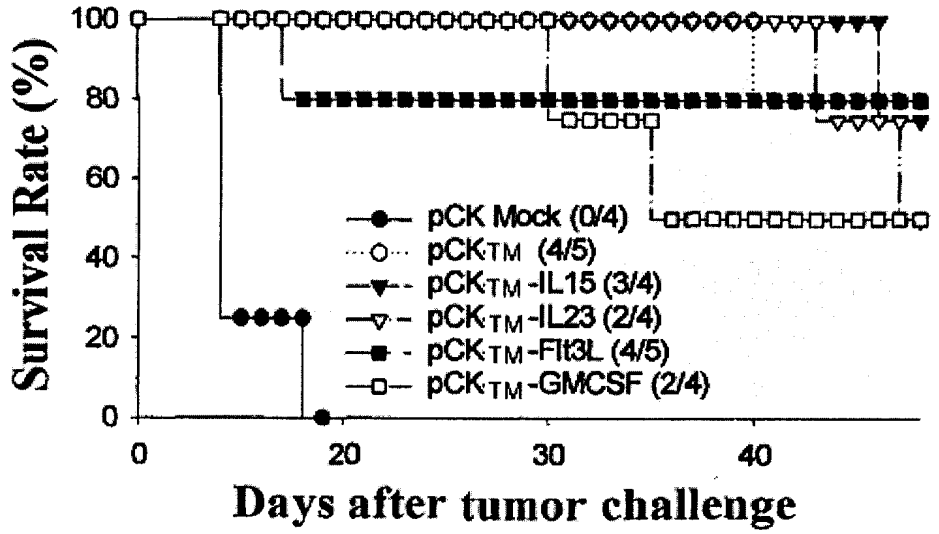


Fig. 15d

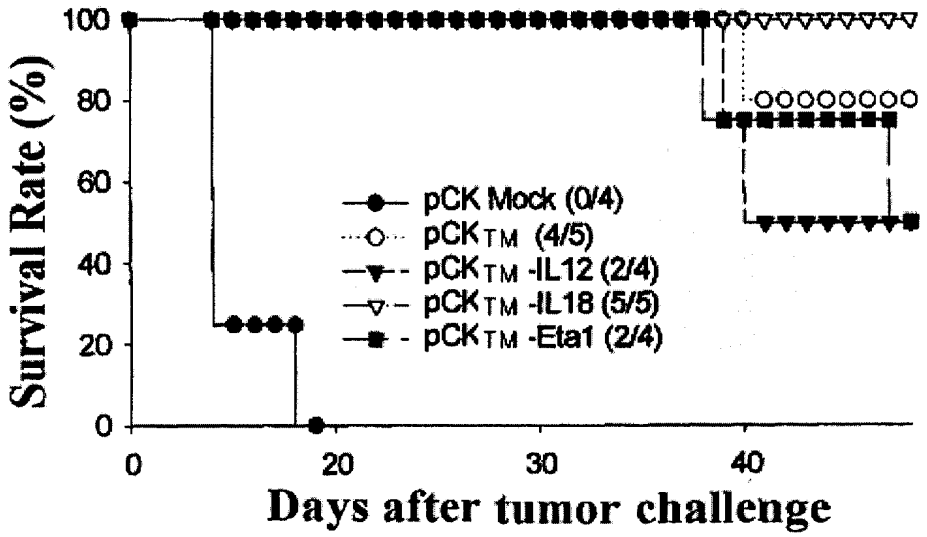


Fig. 16a

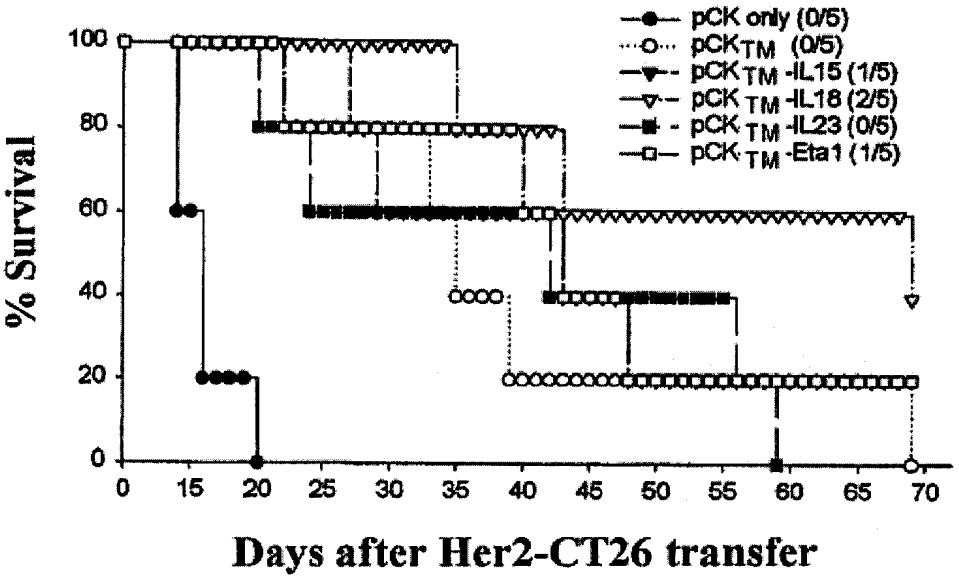
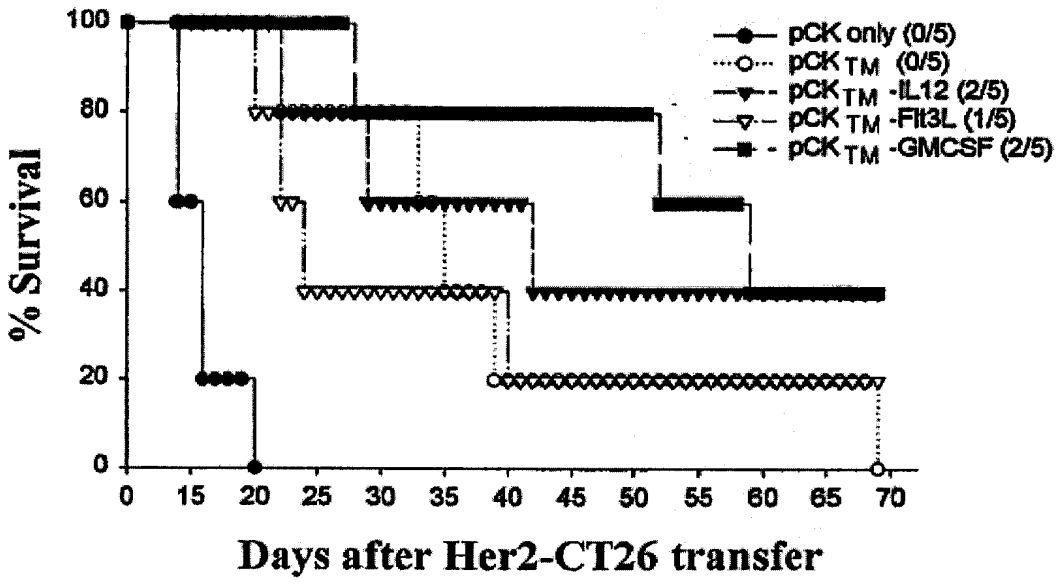


Fig. 16b


100
80
60
40
20
0
% Survival
0
15
20
25
30
35
40
45
50
55
60
65
70
Days after Her2-CT26 transfer
pCK only (0/5)
pCK TM (0/5)
pCK TM -IL12 (2/5)
pCK TM -Flt3L (1/5)
pCK TM -GMCSF (2/5)

SEQUENCE LISTINGS

<110> PANGENOMICS Co., Ltd

<120> Her-2/neu DNA VACCINE HAVING ANTI-CANCER ACTIVITY

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<151> 2002-07-16

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<211> 2052
<212> DNA
<213> human Her-2/neu gene without intracellular region

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aagatccgga ag 2052

<210> 3
<211> 1956
<212> DNA
<213> human Her-2/neu gene without intracellular region and transmembrane domain

<400> 3
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gaactcacct acctgcccac caatgccagc ctgtccttcc tgcaggatat ccaggaggtg 240

cagggctacg tgctcatcgc tcacaaccaa gtgaggcagg tcccactgca gaggctgcgg 300

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cccagcggtg tgaaacctga cctctcctac atgcccatct ggaagtttcc agatgaggag 1860

ggcgcatgcc agccttgccc catcaactgc acccactcct gtgtggacct ggatgacaag 1920

ggctgccccg ccgagcagag agccagccct ctgacg 1956

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<220>
<223> NF6 primer

<400> 4
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<210> 5
<211> 31
<212> DNA
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<220>
<223> NSR1 primer

<400> 5
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<210> 6
<211> 23
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<220>
<223> NF5 primer

<400> 6
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<210> 7
<211> 27
<212> DNA
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<220>
<223> NRM2 primer

<400> 7
ttctagagca gtctccgcat cgtctac 27

<210> 8
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> NSF2 primer

<400> 8
ggcgcgcccc ggcacagaca tgaagctg 28

<210> 9
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> NF3 primer

<400> 9
gccgcagcgg ccgccatgga gctg 24

<210> 10
<211> 1535
<212> DNA
<213> mouse Eta-1 gene

<400> 10
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tattggtgac ttggtggtga tctagtggtg ccaagagtgt gtttgaacct gacaagacat 180

caactgtgcc tcataaaata tgttgcagga ctaactacga ccatgagatt ggcagtgatt 240

tgcttttgcc tgtttggcat tgcctcctcc ctcccggtga aagtgactga ttctggcagc 300

tcagaggaga agctttacag cctgcaccca gatcctatag ccacatggcc ggtgcctgac 360

ccatctcaga agcagaatct ccttgcgcca cagaatgctg tgtcctctga agaaaaggat 420

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agcttggctt atggactgag gtcaaagtct aggagtttcc aggtttctga tgaacagtat 720

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<212> DNA
<213> mouse IL-18 gene

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aaggcacaca aatattgact cactgaaatt ttctctgaga tgtagaaaga ttccataaat 120

tattggtgac ttggtggtga tctagtggtg ccaagagtgt gtttgaacct gacaagacat 180

caactgtgcc tcataaaata tgttgcagga ctaactacga ccatgagatt ggcagtgatt 240

tgcttttgcc tgtttggcat tgcctcctcc ctcccggtga aagtgactga ttctggcagc 300

tcagaggaga agctttacag cctgcaccca gatcctatag ccacatggcc ggtgcctgac 360

ccatctcaga agcagaatct ccttgcgcca cagaatgctg tgtcctctga agaaaaggat 420

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ctagacccta agagtaagga agatgatagg tatctgaaat tccgaatttc tcatgaatta 1080

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ttttttttg ttggtgtgaa taaatcttgt taacg 1535

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<211> 1250
<212> DNA
<213> mouse IL-15

<400> 12
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<210> 13
<211> 699
<212> DNA
<213> mouse Flt3L gene

<400> 13
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ctgagtcctt gcctgcgggg gacacctgac tgttacttca gccacagtcc catctcctcc 120

aacttcaaag tgaagtttag agagttgact gaccacctgc ttaaagatta cccagtcact 180

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<220>
<223> EF1 primer

<400> 14
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<210> 15
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> ER1 primer

<400> 15
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<210> 16
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> 18F1 primer

<400> 16
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<210> 17
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<213> Artificial Sequence

<220>
<223> 18R1 primer

<400> 17
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<210> 18
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<220>
<223> 15F1 primer

<400> 18
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<210> 19
<211> 35
<212> DNA
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<220>
<223> 15R1 primer

<400> 19
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<210> 20
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<213> Artificial Sequence

<220>
<223> FF1 primer

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<210> 21
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<223> FR1 primer

<400> 21
ttctagacta ctgcctgggc cgag 24

<210> 22
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<212> DNA
<213> IRES sequence from pCK-mIL12

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ccgcttggaa taaggccggt gtgcgtttgt ctatatgtta ttttccacca tattgccgtc 120

ttttggcaat gtgagggccc ggaaacctgg ccctgtcttc ttgacgagca ttcctagggg 180

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cccacctggc gacaggtgcc tctgcggcca aaagccacgt gtataagata cacctgcaaa 360

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atctgatctg gggcctcggg gcacatgctt tacatgtgtt tagtccaggt taaaaaaacg 540

tctaggcccc ccgaaccacg gggacgtggt tttcctttga aaaacacgat aataccatgg 600

<210> 23
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> IRES-F1 primer

<400> 23
gcggccgcga taagcttgat atcgaattcc g 31

<210> 24
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> IRES-R1 primer

<400> 24
ctcgagtatt atcgtgtttt tcaaagg 27